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(71) Applicant (for all designated States except US): GENERAL ATOMICS [US/US]; 3550 General Atomics Court, San Diego, CA 92121-1194 (US).

(72) Inventor; and
(75) Inventor/Applicant (for US only): YUAN, Chong-Sheng [CN/US]; 3590 Torrey View Court, San Diego, CA 92130 (US).

(74) Agents: CHEN, Peng et al.; Morrison & Foerster LLP, 2000 Pennsylvania Avenue N.W., Washington, DC 20006-1888 (US).
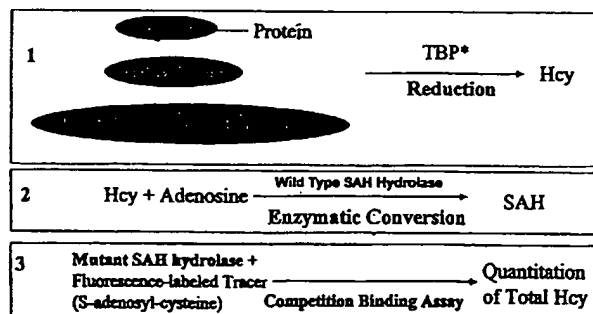
(54) Title: METHODS AND COMPOSITIONS FOR ASSAYING ANALYTES

* tri-n-butylphosphine

(57) Abstract: Compositions and methods for assaying analytes, preferably, small molecule analytes are provided. Assay methods employ, in place of antibodies or molecules that bind to target analytes or substrates, modified enzymes, called substrate trapping enzymes. These modified enzymes retain binding affinity or have enhanced binding affinity for a target substrate or analyte, but have attenuated catalytic activity with respect to that substrate or analyte. The modified enzymes are provided. In particular, mutant S-adenosylhomocysteine (SAH) hydrolases, substantially retaining binding affinity or having enhanced binding affinity for homocysteine or S-adenosylhomocysteine but having attenuated catalytic activity, are provided. Conjugates of the modified enzymes and a facilitating agent, such as agents that aid in purification or linkage to a solid support are also provided.

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METHODS AND COMPOSITIONS FOR ASSAYING ANALYTES

Related Applications

This application is related to U.S. application Serial No. 09/347,878, filed July 6, 1999, entitled "METHODS AND COMPOSITIONS FOR ASSAYING ANALYTES" and U.S. application Serial No. 09/457,205, filed December 6, 1999, entitled "METHODS AND COMPOSITIONS FOR ASSAYING ANALYTES." The subject matter of the above U.S. applications is incorporated in its entirety.

Field of the Invention

The present invention relates to compositions and methods for assaying analytes, preferably, small molecule analytes. More particularly, assay methods that employ, in place of antibodies, modified enzymes that retain binding affinity or have enhanced binding affinity, but that have attenuated catalytic activity, are provided. The modified enzymes and fusion proteins containing the modified enzymes are also provided.

Background of the Invention

Methods for assaying analytes have wide applications. Many analytes including small molecule analytes are essential components and/or participants of biological systems and processes. Methods for assaying these analytes can be used in monitoring the biological systems/processes, or prognosis or diagnosis of diseases or disorders caused by deficiencies and/or imbalances of the analytes. For instance, homocysteine (Hcy), a thiolated amino acid; folic acid, an organic acid; and cholesterol, a lipid are all important prognostic and diagnostic markers for a wide range of cardiovascular diseases. Vitamins are important prognostic and diagnostic markers for various vitamin deficient diseases or disorders. Glucose, a monosaccharide, is a diagnostic marker for numerous glycemic conditions such as diabetic mellitus. Ethanol, an alcohol, is important in monitoring liquor consumption and potential liver damage. Bile acids or bile salts are important prognostic and diagnostic markers for certain cancers such as colon cancer. Monitoring uric acid is important because abnormally high concentration of uric acid is the diagnostic marker and cause of hyperuricemia leading to gout, which is very painful and can cause damage to the kidney. In addition to these prognostic and diagnostic uses, methods for assaying analytes have applications in other agricultural, industrial

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or environmental protection processes where determining the presence, location and amount of the analytes is critical.

Assays for Homocysteine

Homocysteine (Hcy) is a thiol-containing amino acid formed from methionine during S-
5   adenosylmethionine-dependent transmethylation reactions. Intracellular Hcy is remethylated to methionine, or is irreversibly catabolized in a series of reactions to form cysteine. Intracellular Hcy is exported into extracellular fluids such as blood and urine, and circulates mostly in oxidized form, and mainly bound to plasma protein (Refsum, *et al.*, *Annu. Rev. Medicine*, 49:31-62 (1998)). The amount of Hcy in plasma and urine reflects the balance between Hcy
10   production and utilization. This balance may be perturbed by clinical states characterized by genetic disorders of enzymes involved in Hcy transsulfuration and remethylation (*e.g.*, cystathionine ß-synthase and $N^{5,10}$-methylenetetrahydrofolate reductase or dietary deficiency of vitamins (*e.g.*, vitamin B_6, B_{12} and folate) involved in Hcy metabolism (Baual, *et al.*, *Cleveland Clinic Journal of Medicine*, 64:543-549 (1997)). In addition, plasma Hcy levels may also be
15   perturbed by some medications such as anti-folate drugs (*e.g.*, methotrexate) used for treatments of cancer or arthritis (Foody, *et al.*, *Clinician Reviews*, 8:203-210 (1998)).

Severe cases of homocysteinemia are caused by homozygous defects in genes encoding for enzymes involved in Hcy metabolisms. In such cases, a defect in an enzyme involved in either Hcy remethylation or transsulfuration leads to as much as 50-fold elevations of Hcy in
20   the blood and urine. The classic form of such a disorder, congenital homocysteinemia (Hcyemia), is caused by homozygous defects in the gene encoding cystathionine ß-synthase (CBS). These individuals suffer from thromboembolic complications at an early age, which result in stroke, myocardial infarction, renovascular hypertension, intermittent claudication, mesenteric ischemic, and pulmonary embolism. Such patients may also exhibit mental
25   retardation and other abnormalities resembling ectopia lentis and skeletal deformities (Perry T., *Homocysteine: Selected aspects in Nyham W.L. ed. Heritable disorders of amino acid metabolism*. New York, John Wiley & Sons, pp. 419-451 (1974)). It is also known that elevated Hcy levels in pregnant women is related to birth defects of children with neurotube closures (Scott, *et al.*, *"The etiology of neural tube defects"* in *Graham, I., Refsum, H.,*
30   *Rosenberg, I.H., and Ureland P.M. ed. "Homocysteine metabolism: from basic science to clinical medicine"* Kluwer Academic Publishers, Boston, pp. 133-136 (1995)). Thus, the diagnostic utility of Hcy determinations has been well documented in these clinical conditions.

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It has been demonstrated that even mild or moderately elevated levels of Hcy also increase the risk of atherosclerosis of the coronary, cerebral and peripheral arteries and cardiovascular disease (Boushey, *et al.*, *JAMA*, 274:1049-1057 (1995)). The prevalence of Hcyemia was shown to be 42%, 28%, and 30% among patients with cerebral vascular disease,

5 peripheral vascular disease and cardiovascular disease, respectively (Moghadasian, *et al.*, *Arch. Intern. Med.*, 157:2299-2307 (1997)). A meta-analysis of 27 clinical studies calculated that each increase of 5 μM in Hcy level increases the risk for coronary artery disease by 60% in men and by 80% in women, which is equivalent to an increase of 20 mg/dl^{-1} (0.5 mmol/dl^{-1}) in plasma cholesterol, suggesting that Hcy, as a risk factor, is as strong as cholesterol in the

10 general population. Results from these clinical studies concluded that hyperhomocysteinemia is an emerging new independent risk factor for cardiovascular disease, and may be accountable for half of all cardiovascular patients who do not have any of the established cardiovascular risk factors (*e.g.*, hypertension, hypercholesterolemia, cigarette smoking, diabetes mellitus, marked obesity and physical inactivity).

15 Mild homocysteinemia is mainly caused by heterozygosity of enzyme defects. A common polymorphism in the gene for methylenetetrahydrofolate reductase appears to influence the sensitivity of homocysteine levels to folic acid deficiency (Boers, *et al.*, *J. Inher. Metab. Dis.*, 20:301-306 (1997)). Moreover, plasma homocysteine levels are also significantly increased in heart and renal transplant patients (Ueland, *et al.*, *J. Lab. Clin. Med.*, 114:473-501

20 (1989)), Alzheimer patients(Jacobsen, *et al.*, *Clin. Chem.*, 44:2238-2239 (1998)), as well as in patients of non-insulin-dependent diabetes mellitus (Ducloux, *et al.*, *Nephrol. Dial. Transplantl*, 13:2890-2893 (1998)). The accumulating evidence linking elevated homocysteine with cardiovascular disease has prompted the initiation of double-blind, randomized and placebo controlled multicenter clinical trials to demonstrate the efficacy of lowering plasma

25 Hcy in preventing or halting the progress of vascular disease (Diaz-Arrastia, *et al.*, *Arch. Neurol.*, 55:1407-1408 (1998)). Determination of plasma homocysteine levels should be a common clinical practice.

As a risk factor for cardiovascular disease, the determination of total plasma Hcy levels (reduced, oxidized and protein-bound) has been recommended in clinical setting (Hornberger,

30 *et al.*, *American J. of Public Health*, 88:61-67 (1998)). Since 1982, several methods for determining total plasma Hcy have been described (Mansoor, *et al.*, *Anal. BioChem.*, 200:218-229 (1992); Steir, *et al.*, *Arch. Intern. Med.*, 158:1301-1306 (1998); Ueland, *et al.*, *Clin. Chem.*, 39:1764-1779 ()1993); and Ueland, *et al.*, *"Plasma homocysteine and cardiovascular disease"*

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in Francis, R.B.Jr.eds. Atherosclerotic Cardiovascular Disease, Hemostasis, and Endothelial Function. New York, Marcel Dokker, pp. 183-236 (1992); see, also, Ueland, *et al.*, *"Plasma homocysteine and cardiovascular disease" in Francis, R.B.Jr.eds. Atherosclerotic Cardiovascular Disease, Hemostasis, and Endothelial Function.* New York, Marcel Dokker,

5   pp. 183-236 (1992)). The assay of total Hcy in plasma or serum is complicated by the fact that 70% of plasma Hcy is protein-bound and 20-30% exists as free symmetric or mostly asymmetric mixed disulfides. Free reduced Hcy exists in only trace amounts (Stehouwer, *et al.*, *Kidney International,* 55:308-314 (1999)).

Most of the methods require sophisticated chromatographic techniques such as HPLC,

10   capillary gas chromatography, or mass spectrometry (GC/MS) to directly or indirectly (*e.g.*, enzymatic conversion of Hcy to SAH (S-adenosylhomocysteine) by SAH hydrolase followed by HPLC or TLC separation) measure Hcy. Radioenzymatic conversion of Hcy to radiolabeled SAH by SAH hydrolase prior to TLC separation has also been used. A feature common to these methods includes the following four steps: (1) reduction of oxidized Hcy to reduced Hcy;

15   (2) precolumn derivitization or enzymic conversion to SAH; (3) chromatographic separation; and (4) detection of the Hcy derivative or SAH. In these assays, chromatographic separation, which is often time-consuming and cumbersome to perform, is a common key step of these methods. More particularly, these methods require highly specialized and sophisticated equipment and well-trained analytic specialists. The use of such equipment is generally not

20   well-accepted in routine clinical laboratory practice.

Immunoassays for Hcy that use a monoclonal antibody against SAH (Araki, *et al., J. Chromatog.,* 422:43-52 (1987) are also known. These assays are based upon conversion of Hcy to SAH, which is then detected by a monoclonal antibody. Monoclonal antibody against albumin-bound Hcy has been developed for determination of albumin-bound Hcy (Stabler, *et*

25   *al., J. Clin. Invest.,* 81:466-474 (1988)), which is the major fraction of total plasma Hcy. Other immunological protocols are also available (see, *e.g.*, U.S. Patent No. 5,885,767 and U.S. Patent No. 5,631,127) Though immunoassays avoid a time-consuming chromatographic separation step and are amenable to automation, production of monoclonal antibody is expensive, somewhat unpredictable, and often requires secondary or even tertiary antibodies

30   for detection.

Hence, in general, methods for assaying analytes suffer from several deficiencies. First, for many analytes, specific binding partners are not readily available and this lack of specific binding partner often compromises the specificity of the assay method. Although such

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deficiencies may be overcome by generating antibodies for macromolecule analytes, generating

antibodies, especially monoclonal antibodies with the desired specificity and uniformity, is

often time consuming and expensive. In addition, for many small molecule analytes, the option

of generating antibodies is often not available because small molecules are poor antigens.

5   Generation of antibodies against small molecules usually requires conjugation of the small

molecules to macromolecules, and this often makes the antibody screening more tedious and

laborious. Second, many methods for assaying analytes, especially small molecule analytes,

involve chemical derivations and chromatographic separation which can be time consuming.

Third, many such assay methods use sophisticated and expensive analytical equipment such as

10   HPLC's and GC/MS. Hence there is a need for rapid simpler assays that address these

deficiencies.

It is an object herein to provide assays for detecting analytes. It is also an object herein

to provide such an assay for quantifying and/or detecting homocysteine in body fluids and body

tissues.

15   SUMMARY OF THE INVENTION

Assays, particularly assays that are based on immunoassay formats, but that employ

mutant analyte-binding enzymes that, substantially retain binding affinity or have enhanced

binding affinity for desired analytes or an immediate analyte enzymatic conversion products

but have attenuated catalytic activity, are provided. In place of antibodies, these assays use

20   modified enzymes that retain binding affinity or have enhanced binding affinity, but have

attenuated catalytic activity. These methods are designated substrate trapping methods; and the

modified enzymes, are designated as "substrate trapping enzymes." The substrate trapping

enzymes (also designated pseudoantibodies) and methods for preparing them are also provided.

These substrate trapping enzymes are intended to replace antibodies, monoclonal, polyclonal or

25   any mixture thereof, in reactions, methods, assays and processes in which an antibody

(polyclonal, monoclonal or specific binding fragment thereof) is a reactant. They can also act

as competitive inhibitors with analytes for binding to entities such as receptors and other anti-

ligands and other analytes. Hence, they can be used in competitive binding assays in place of,

for example, receptor agonists or modulators of receptor activity, and for assays that monitor

30   drugs.

Any process or method, particularly immunoassays or assays in which an antibody aids

in detection of a target analyte, can be modified as described herein, by substituting a substrate

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trapping enzyme for the antibody used in the process or method. The substrate trapping
enzymes can be prepared by any method known to those of skill in the art by which the
catalytic activity of an enzyme is substantially attenuated or eliminated, without affecting or
without substantially reducing the binding affinity of the resulting modified enzyme for an

5   analyte. Other methods in which antibodies may be substituted by mutant analyte binding
enzymes, include but are not limited to, affinity purification methods, and methods in which the
mutant enzymes replace neutralizing antibodies.

The methods are particularly useful for detecting analytes indicative of metabolic
conditions, inborn errors of metabolism, such as hypothyroidism, galactosemia,

10   phenylketonuria (PKU), and maple syrup urine disease; disease markers, such as glucose
levels, cholesterol levels, Hcy levels and other such parameters in body fluid and tissue samples
from mammals, including humans. The methods also include methods for detecting
contaminants in food, for testing foods to quantitate certain nutrients, for screening blood. The
assays readily can be automated. In addition, the assays can be adapted for use in point of care

15   systems and in home test kits. For example, blood test point of care systems can be adapted for
measuring homocysteine levels using the mutant enzymes provided herein. Home test kits may
also be adapted for use with the methods and mutant enzymes provided herein.

Accordingly, methods in which an antibody is a reactant, wherein the improvement is
replacement of the antibody with a substrate trapping enzyme, as defined herein, are provided.

20   The methods may also rely on competitive binding of the modified enzyme for a target analyte.

In another embodiment, a method is provided for assaying an analyte, preferably a
small molecule analyte, in a sample by: a) contacting the sample with a mutant analyte-binding
enzyme, the mutant enzyme substantially retains its binding affinity or has enhanced binding
affinity for the analyte or an immediate analyte enzymatic conversion product but has

25   attenuated catalytic activity; and b) detecting binding between the analyte or the immediate
analyte enzymatic conversion product and the mutant analyte-binding enzyme.

The small molecule analyte to be assayed can be any analyte, including organic and
inorganic molecules. Typically the small molecule to be assayed has a molecular weight that is
about or less than 10,000 daltons. Preferably, the small molecule has a molecular weight that is

30   about or less than 5,000 daltons.

Inorganic molecules include, but are not limited to, an inorganic ion such as a sodium,
a potassium, a magnesium, a calcium, a chlorine, an iron, a copper, a zinc, a manganese, a
cobalt, an iodine, a molybdenum, a vanadium, a nickel, a chromium, a fluorine, a silicon, a tin,

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a boron or an arsenic ion. Organic molecules include, but are not limited to, an amino acid, a peptide, typically containing less than about 10 amino acids, a nucleoside, a nucleotide, an oligonucleotide, typically containing less than about 10 nucleotides, a vitamin, a monosaccharide, an oligosaccharide containing less than 10 monosaccharides or a lipid.

5   The amino acids, include, but are not limited to, D- or L-amino-acids, including the building blocks of naturally-occurring peptides and proteins including Ala (A), Arg (R), Asn (N), Asp (D), Cys (C), Gln (Q), Glu (E), Gly (G), His (H), Ile (I), Leu (L), Lys (K), Met (M), Phe (F), Pro (P) Ser (S), Thr (T), Trp (W), Tyr (Y) and Val (V).

Nucleosides, include, but are not limited to, adenosine, guanosine, cytidine, thymidine 10 and uridine. Nucleotides include, but are not limited to, AMP, GMP, CMP, UMP, ADP, GDP, CDP, UDP, ATP, GTP, CTP, UTP, dAMP, dGMP, dCMP, dTMP, dADP, dGDP, dCDP, dTDP, dATP, dGTP, dCTP and dTTP.

Vitamins, include, but are not limited to, water-soluble vitamins such as thiamine, riboflavin, nicotinic acid, pantothenic acid, pyridoxine, biotin, folate, vitamin B_{12} and ascorbic 15 acid, fat-soluble vitamins such as vitamin A, vitamin D, vitamin E, and vitamin K.

Monosaccharides, include but are not limited to, D- or L-monosaccharides and whether aldoses or ketoses. Monosaccharides include, but are not limited to, triose, such as glyceraldehyde, tetroses such as erythrose and threose, pentoses such as ribose, arabinose, xylose, lyxose and ribulose, hexoses such as allose, altrose, glucose, mannose, gulose, idose, 20 galactose, talose and fructose and heptose such as sedoheptulose.

Lipids, include, but are not limited to, triacylglycerols such as tristearin, tripalmitin and triolein, waxes, phosphoglycerides such as phosphatidylethanolamine, phosphatidylcholine, phosphatidylserine, phosphatidylinositol and cardiolipin, sphingolipids such as sphingomyelin, cerebrosides and gangliosides, sterols such as cholesterol and stigmasterol and sterol fatty acid 25 esters. The fatty acids can be saturated fatty acids such as lauric acid, myristic acid, palmitic acid, stearic acid, arachidic acid and lignoceric acid, or can be unsaturated fatty acids such as palmitoleic acid, oleic acid, linoleic acid, linolenic acid and arachidonic acid.

In an exemplary embodiment, mutant S-adenosylhomocysteine (SAH) hydrolases, substantially retaining binding affinity or having enhanced binding affinity for homocysteine 30 (Hcy) or SAH but having attenuated catalytic activity, are provided. Also provided are methods, combinations, kits and articles of manufacture for assaying analytes, preferably small molecule analytes such as inorganic ions, amino acids (e.g., homocysteine), peptides, nucleosides, nucleotides, oligonucleotides, vitamins, monosaccharides (e.g., glucose),

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oligosaccharides, lipids (*e.g.*, cholesterol), organic acids (*e.g.*, folate species, bile acids and uric acids).

In another embodiment, provided herein are purified mutant SAH hydrolases, the mutant SAH hydrolases substantially retain their binding affinity or have enhanced binding
5   affinity for homocysteine (Hcy) or SAH but have attenuated catalytic activity.

Examples of such mutant SAH hydrolases include those in which the attenuated catalytic activity is caused by mutation(s) in the mutant SAH hydrolase's binding site for NAD$^+$, or mutation(s) in the mutant SAH hydrolase's catalytic site or a combination thereof; those that have attenuated 5'-hydrolytic activity but substantially retain the 3'-oxidative activity;
10   those that irreversibly bind SAH; those that have a Km for SAH that is about or less than 10.0 μM; those that have a Kcat for SAH that is about or less than 0.1 S^{-1}; those that have one or more insertion, deletion, or point mutation(s); those that are derived from the sequence of amino acids set forth in SEQ ID No. 1 or encoded by the sequence of nucleotides set forth in SEQ ID No. 2 and have one or, preferably at least two or more mutations selected from Phe
15   302 to Ser (F302S), Lys 186 to Ala (K186A), His 301 to Asp (H301D), His 353 to Ser (H353S), Arg 343 to Ala (R343A), Asp 190 to Ala (D190A), Phe 82 to Ala (F82A), Thr 157 to Leu (T157L), Cys 195 to Asp (C195D), Asn 181 to Asp (N181D), and deletion of Tyr 432 (Δ432); or those that are derived from the sequence of amino acids set forth in SEQ ID No. 1 or encoded by the sequence of nucleotides set forth in SEQ ID No. 2 and have a combination of
20   Arg 431 to Ala (R431A) and Lys 426 to Arg (K426R) mutations; or any that hybridize under conditions of low, more preferably moderate, most preferably high, stringency along their full-length and have a Km at least about 10%, more preferably at least about 50% of the Km of the wildtype enzyme for the analyte or substrate, but having substantially attenuated catalytic activity to the coding portion of the sequence of nucleotides set forth in SEQ ID No. 1 or
25   encoding the sequence of amino acids set forth in SEQ ID No. 2.

Isolated nucleic acid fragments encoding the above-described mutant SAH hydrolases, preferably in the form of plasmid or expression vectors, are also provided. Recombinant host cells, especially recombinant bacterial cells, yeast cells, fungal cells, plant cells, insect cells and animal cells, containing the plasmids or vectors are further provided. Methods for producing
30   the mutant SAH hydrolases using the recombinant host cells are further provided.

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Assays for homocysteine and metabolically related analytes

Assays for homocysteine, which as noted above, is a risk factor for cardiovascular disease and other diseases, are provided herein.

Homocysteine

5   In these embodiments, the small molecule to be assayed is homocysteine (Hcy) and the mutant analyte-binding enzymes are mutant Hcy-binding enzymes that substantially retain their binding affinity or that have enhanced binding affinity for Hcy or an immediate Hcy enzymatic conversion product but have attenuated catalytic activity.

Mutant Hcy-binding enzymes that can be used in the assay include those in which the
10   attenuated catalytic activity is caused by mutation in the mutant enzyme's binding site for its co-enzyme or for a non-Hcy substrate, or mutation in the mutant enzyme's catalytic site or a combination thereof.

In another embodiment, the mutant enzyme is a mutant cystathionine ß-synthase and the attenuated catalytic activity is caused by mutation in the mutant cystathionine ß-synthase's
15   catalytic site, its binding site for pyridoxal 5'-phosphate or L-serine, or a combination thereof.

In another embodiment, the mutant enzyme is a mutant methionine synthase and the attenuated catalytic activity is caused by mutation in the mutant methionine synthase's catalytic site, its binding site for vitamin B_{12} or 5-methyltetrahydrofolate (5-CH$_3$-THF), or a combination thereof. More preferably, the mutant methionine synthase is an *E. coli.* methionine synthase,
20   the mutant methionine synthase has one or more of the following mutations: His759Gly, Asp757Glu, Asp757Asn, and Ser810Ala.

In another embodiment, the mutant enzyme is a mutant methioninase and the attenuated catalytic activity is caused by mutation in the mutant methionine synthase's catalytic site, its binding site for a compound with the formulae of R'SH, in which R'SH is a substituted thiol,
25   where R is preferably alkyl, preferably lower alkyl (1 to 6 carbons, preferably 1 to 3 carbons, in a straight or branched chain), heteroaryl, where the heteroatom is O, S or N, or aryl, which is substituted, such as with alkyl, preferably lower alkyl, or hal, or unsubstituted, preferably aryl or heteroaryl with one ring or two to three fused rings, preferably with about 4 to 7 members in each ring, or combinations of any of the above.

30   In a preferred embodiment, the mutant enzyme is a mutant SAH hydrolase, where the mutant SAH hydrolase substantially retains its binding affinity or has enhanced binding affinity for Hcy or SAH but has attenuated catalytic activity. Examples of such mutant SAH

hydrolases that can be used in the assay include those in which the attenuated catalytic activity is caused by mutation(s) in the mutant SAH hydrolase's binding site for NAD^+, or mutation(s) in the mutant SAH hydrolase's catalytic site or a combination thereof; those that have attenuated 5'-hydrolytic activity but substantially retains its 3'-oxidative activity; those that
5   irreversibly bind SAH; those that have a Km for SAH that is about or less than 10.0 μM; those that have a Kcat for SAH that is about or less than 0.1 S^{-1}; those that have one or more insertion, deletion, or point mutation(s); those that are derived from the sequence of amino acids set forth in SEQ ID No. 1 or encoded by the sequence of nucleotides set forth in SEQ ID No. 2 but have one or more of the following mutations: Phe 302 to Ser (F302S), Lys 186 to Ala
10   (K186A), His 301 to Asp (H301D), His 353 to Ser (H353S), Arg 343 to Ala (R343A), Asp 190 to Ala (D190A), Phe 82 to Ala (F82A), Thr 157 to Leu (T157L), Cys 195 to Asp (C195D), Asn 181 to Asp (N181D), and deletion of Tyr 432 (Δ432); or those that are derived from a sequence of amino acids set forth in SEQ ID No. 1 or encoded by the sequence of nucleotides set forth in SEQ ID No. 2 and have a combination of Arg 431 to Ala (R431A) and Lys 426 to
15   Arg (K426R) mutations or any that hybridize under conditions of low, more preferably moderate, most preferably high, stringency along their full-length and have a Km at least about 10%, more preferably at least about 50% of the Km of the wildtype enzyme for the analyte or substrate, but having substantially attenuated catalytic activity.

       In one embodiment that uses a mutant SAH hydrolase, oxidized Hcy in the sample is
20   converted into reduced Hcy prior to the contact between the sample and the mutant SAH hydrolase. The oxidized Hcy in the sample is converted into reduced Hcy by a reducing agent, such as, but not limited to, tri-n-butylphosphine (TBP), ß-ME, DTT, dithioerythritol, thioglycolic acid, glutathione, tris (2-carboxyethyl)phosphine, sodium cyanoborohydride, $NaBH_4$, KBH_4 and free metals.
25   In another embodiment that uses a mutant SAH hydrolase, prior to the contact between the sample and the mutant SAH hydrolase, the Hcy in the sample is converted into SAH. More preferably, the Hcy in the sample is converted into SAH by a wild-type SAH hydrolase. Also more preferably, the SAH in the sample is contacted with the mutant SAH hydrolase in the presence of a SAH hydrolase catalysis inhibitor, such as, but are not limited to, neplanocin A or
30   thimersal.

       In another embodiment that uses a mutant SAH hydrolase, prior to the contact between the SAH and the mutant SAH hydrolase, free adenosine is removed or degraded. More

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preferably, free adenosine is degraded by combined effect of adenosine deaminase, purine nucleoside phosphorylase and xanthine oxidase.

In another embodiment that uses a mutant SAH hydrolase, the SAH is contacted with the mutant SAH hydrolase in the presence of a labeled SAH or a derivative or an analog thereof, whereby the amount of the labeled SAH bound to the mutant SAH hydrolase inversely relates to amount of the SAH in the sample. More preferably, the labeled SAH derivative or analog is a fluorescence labeled adenosyl-cysteine.

In another embodiment that uses a mutant SAH hydrolase, the mutant SAH hydrolase is labeled mutant SAH hydrolase. More preferably, the mutant SAH hydrolase is labeled by fluorescence.

In still another embodiment, the mutant enzyme is a mutant betaine-homocysteine methyltransferase and the attenuated catalytic activity is caused by mutation in the mutant betaine-homocysteine methyltransferase's binding site for betaine, its catalytic site, or a combination thereof.

In another embodiment, the Hcy assay is performed in combination with assays for other analytes associated with cardiovascular disease and/or regulation of Hcy levels, such as assays for cholesterol and/or folic acid.

Folate

In another embodiment, the mutant enzyme is a mutant methionine synthase. In this embodiment, the folate species can be a 5,-methyl-tetrahydrofolate, the mutant folate-species-binding enzyme is a mutant methionine synthase, and the attenuated catalytic activity of the mutant methionine synthase is caused by mutation in its catalytic site, its binding site for vitamin B_{12}, Hcy, or a combination thereof.

In another embodiment, the folate species is tetrahydrofolate, the mutant folate-species-binding enzyme is a mutant tetrahydrofolate methyltransferase, and the attenuated catalytic activity of the mutant tetrahydrofolate methyltransferase is caused by mutation in its catalytic site, its binding site for serine, or a combination thereof.

In still another embodiment, the folate species is 5, 10,-methylene tetrahydrofolate, the mutant folate-species-binding enzyme is a mutant methylenetetrahydrofolate reductase, and the attenuated catalytic activity of the methylenetetrahydrofolate reductase is caused by mutation in its catalytic site.

In yet another embodiment, the folate species is 5, 10,-methylene tetrahydrofolate, the mutant folate-species-binding enzyme is a mutant folypolyglutamate synthase, and the attenuated catalytic activity of the folypolyglutamate synthase is caused by mutation in its catalytic site, its binding site for ATP, L-glutamate, Mg^{2+}, or a combination thereof.

In yet another preferred embodiment, the folate species is dihydrofolate, the mutant folate-species-binding enzyme is a mutant dihydrofolate reductase, and the attenuated catalytic activity of the mutant dihydrofolate reductase is caused by mutation in its catalytic site, its binding site for NADH/NADPH, or a combination thereof. More preferably, the mutant dihydrofolate reductase is a *Lactobacillus casei* dihydrofolate reductase having the Arg43Ala or Trp21His mutation (Basran, *et al.*, *Protein Eng.*, 10(7):815-26 91997)).

In yet another embodiment, the folate species is 5, 10,-methylene tetrahydrofolate (5, 10-methylene-FH₄), the mutant folate-species-binding enzyme is a mutant thymidylate synthase, and the attenuated catalytic activity of the mutant thymidylate synthase is caused by mutation in its catalytic site, its binding site for dUMP, or a combination thereof. More preferably, the mutant thymidylate synthase is a human thymidylate synthase having a mutation selected from Tyr6His, Glu214Ser, Ser216Ala, Ser216Leu, Asn229Ala and His199X, where X is any amino acid that is not His (Schiffer, *et al.*, *Biochemistry*, 34(50):16279-87 (1995); Steadman, *et al.*, *Biochemistry*, 37:7089-7095 (1998); Williams, *et al.*, *Biochemistry*, 37(20):7096-102 (1998); Finer-Moore, *et al.*, *J. Mol. Biol.*, 276(1):113-29 (1998); and Finer-Moore, *et al.*, *Biochemistry*, 35(16):5125-36 (1996)). Also more preferably, the mutant thymidylate synthase is an *E. coli* thymidylate synthase having an Arg126Glu mutation (Strop, *et al.*, *Protein Sci.*, 6(12):2504-11 (1997)) or a *Lactobacillus casei* thymidylate synthase having a V316Am mutation (Carreras, *et al.*, *Biochemistry*, 31(26):6038-44 (1992)).

Cholesterol

In another embodiment, the analyte is cholesterol and the mutant analyte-binding enzyme is a mutant cholesterol-binding enzyme, where the mutant enzyme substantially retains its binding affinity or has enhanced binding affinity for cholesterol but has attenuated catalytic activity. In a preferred embodiment, the mutant cholesterol-binding enzyme is a mutant cholesterol esterase, and the attenuated catalytic activity of the mutant cholesterol esterase is caused by mutation in its catalytic site, its binding site for H_2O or a combination thereof. More preferably, the cholesterol esterase is a pancreatic cholesterol esterase having a Ser194Thr or Ser194Ala mutation (DiPersio, *et al.*, *J. Biol. Chem.*, 265(28):16801-6 (1990)). In another

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preferred embodiment, the mutant cholesterol-binding enzyme is a mutant cholesterol oxidase, and the attenuated catalytic activity of the mutant cholesterol oxidase is caused by mutation in its catalytic site, its binding site for O_2 or a combination thereof. More preferably, the cholesterol oxidase is a *Brevibacterium sterolicum* cholesterol oxidase having a His447Asn or
5   His447Gln mutation (Yue, *et al.*, *Biochemistry*, 38(14):4277-86 (1999)).

Bile acid (salt)

In still another spe.. ..u embodiment, the small molecule analyte is a bile acid (salt) and the mutant analyte-binding enzyme is a mutant bile-acid (salt)-binding enzyme, the mutant enzyme substantially retains its binding affinity or has enhanced binding affinity for the bile
10 acid (salt) but has attenuated catalytic activity. Preferably, the mutant bile-acid (salt)-binding enzyme is a mutant 3-α-hydroxy steroid dehydrogenase, and the attenuated catalytic activity of the mutant 3-α-hydroxy steroid dehydrogenase is caused by mutation in its catalytic site, its binding site for NAD^+ or a combination thereof.

Assays for disorders associated with glucose metabolism

15 In yet another specific embodiment, the small molecule analyte is glucose and the mutant analyte-binding enzyme is a mutant glucose-binding enzyme, the mutant enzyme substantially retains its binding affinity or has enhanced binding affinity for glucose but has attenuated catalytic activity. Preferably, the mutant glucose-binding enzyme is a *Clostridium thermosulfurogenes* glucose isomerase having a mutation selected from His101Phe,
20 His101Glu, His101Gln, His101Asp and His101Asn (Lee, *et al.*, *J. Biol. Chem.*, 265(31):19082-90 (1990)). Also preferably, the mutant glucose-binding enzyme is a mutant hexokinase or glucokinase, and the attenuated catalytic activity of the mutant hexokinase or glucokinase is caused by mutation in its catalytic site, its binding site for ATP or Mg^{2+}, or a combination thereof. Further preferably, the mutant glucose-binding enzyme is a mutant glucose oxidase,
25 and the attenuated catalytic activity of the mutant glucose oxidase is caused by mutation in its catalytic site, its binding site for H_2O or O_2, or a combination thereof. Any disorders associated with glucose metabolism may be monitored or assessed.

Ethanol

In yet another specific embodiment, the small molecule analyte is ethanol and the
30 mutant analyte-binding enzyme is a mutant ethanol-binding enzyme, the mutant enzyme

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substantially retains its binding affinity or has enhanced binding affinity for ethanol but has attenuated catalytic activity. Preferably, the mutant ethanol-binding enzyme is a mutant alcohol dehydrogenase, and the attenuated catalytic activity of the mutant alcohol dehydrogenase is caused by mutation in its catalytic site, its binding site for NAD^+ or Zn^{2+}, or a

5   combination thereof. More preferably, the mutant alcohol dehydrogenase is a human liver alcohol dehydrogenase having a His51Gln mutation (Ehrig, *et al.*, *Biochemistry*, 30(4):1062-8 (1991)). Also more preferably, the mutant alcohol dehydrogenase is a horse liver alcohol dehydrogenase having a Phe93Trp or Val203Ala mutation (Bahnson, *et al.*, *Proc. Natl. Acad. Sci.*, 94(24):12797-802 (1997); Colby, *et al.*, *Biochemistry*, 37(26):9295-304 (1998)).

10   Assays for disorders, such as gout, associated with uric acid metabolism
                In another exemplary embodiment, the small molecule analyte is uric acid and the mutant analyte-binding enzyme is a mutant uric-acid-binding enzyme, the mutant enzyme substantially retains its binding affinity or has enhanced binding affinity for uric acid but has attenuated catalytic activity. Preferably, the mutant uric-acid-binding enzyme is a mutant urate

15   oxidase, and the attenuated catalytic activity of the mutant urate oxidase is caused by mutation in its catalytic site, its binding site for O_2, H_2O, or copper ion, or a combination thereof. More preferably, the mutant urate oxidase is a rat urate oxidase having a mutation selected from H127Y, H129Y and F131S (Chu, *et al.*, *Ann. N.Y. Acad. Sci.*, 804:781-6 (1996)).
                In all embodiments, the sample being assayed typically is a body fluid or tissue,

20   including, but not limited to blood, urine, cerebral spinal fluid, synovial fluid, amniotic fluid, and tissue samples, such as biopsied tissues. Preferably, the body fluid is blood or urine. More preferably, the blood sample is further separated into a plasma or sera fraction.
                Further provided herein are combinations that include: a) a mutant analyte-binding enzyme, the mutant enzyme substantially retains its binding affinity or has enhanced binding

25   affinity for the analyte or an immediate analyte enzymatic conversion product but has attenuated catalytic activity; and b) reagents and or other means for detecting binding between the analyte or the immediate analyte enzymatic conversion product with the mutant analyte-binding enzyme. Preferably, binding between the analyte or the immediate analyte enzymatic conversion product with the mutant analyte-binding enzyme is detected using a labeled analyte,

30   a labeled immediate analyte enzymatic conversion product, or a derivative or an analog thereof, or a labeled mutant analyte-binding enzyme. Also preferably, the combination where the analyte is Hcy further also includes reagents for detecting cholesterol and/or folic acid.

Kits and articles of manufacture that include the above combinations and optional instructions for performing the assay of interest are provided. Articles of manufacture that contain the mutant enzymes with a label indicating the assay in which the enzyme is used, and also packaging material that contains the enzyme.

5   Other diagnostic and prognostic assays

Other assays include, but are not limited to, diagnostic and prognostic assays in which markers, especially small molecule markers associated with various diseases, defects, conditions or drugs are monitored. Exemplary small molecule analytes and mutant enzymes include, but are not limited to, any of the following in which:

10   the small molecule analyte is creatinine and the mutant analyte-binding enzyme is a mutant creatinine amidohydrolase,

the small molecule analyte is serotonin and the mutant analyte-binding enzyme is a mutant serotonin N-acetyltransferase,

the small molecule analyte is hyaluronic acid and the mutant analyte-binding enzyme is

15   a mutant hyaluronidase,

the small molecule analyte is catecholamine and the mutant analyte-binding enzyme is a mutant catechol O-methyltransferase,

the small molecule analyte is homovanillic acid and the mutant analyte-binding enzyme is a mutant monoamine oxidase,

20   the small molecule analyte is vanilylmandelic acid and the mutant analyte-binding enzyme is a mutant dopamine ß-hydroxylase,

the small molecule analyte is cyclosporin A and the mutant analyte-binding enzyme is a mutant calcineurine or cyclophilin,

the small molecule analyte is mycophenoric acid and the mutant analyte-binding

25   enzyme is a mutant inosine monophosphate dehydrogenase,

the small molecule analyte is leflunomide and the mutant analyte-binding enzyme is a mutant dihydroorotate dehydrogenase,

the small molecule analyte is N-acetylprocainamide and the mutant analyte-binding enzyme is a mutant procainamide N-acetyltransferase,

30   the small molecule analyte is selected from the group consisting of fluvastatin, lovastatin, provastatin, simvastatin and atorvastatin and the mutant analyte-binding enzyme is a mutant hydroxymethylglutaryl-CoA reductase.

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Conjugates, preparation and uses thereof

Also provided herein are conjugates of the mutant analyte binding enzymes and an additional portion, referred to herein as a facilitating agent, linked directly or indirectly via a linker to the analyte binding protein. The facilitating agent is linked directly or indirectly,

5   typically covalently or via ionic interactions, and is selected to facilitate, for example: i) affinity isolation or purification of the conjugate, such as a tag that specifically binds to an immobilized receptor; ii) immobilization, such as attachment of the conjugate to a surface; or iii) detection of the conjugate, such as a linker.

Hence the conjugates provided herein contain the following components: (mutant

10 analyte binding enzyme)$_n$, (L)$_q$, and (facilitating agent)$_m$ in which at least one mutant analyte binding enzyme is linked directly or via one or more linkers (L) to at least one facilitating agent. L refers to a linker. Any suitable association among the elements of the conjugate is contemplated as long as the resulting conjugate substantially retains binding affinity or has enhanced binding affinity for desired analytes or immediate analyte enzymatic conversion

15 products but has attenuated catalytic activity, and the facilitating agent retains the desired activity.

The variables n and m are integers of 1 or greater and q is 0 or any integer. The variables n, q and m are selected such that the resulting conjugate interacts with the targeted receptor and a targeted agent is internalized by a cell to which it has been targeted. Typically n

20 is between 1 and 3; q is 0 or more, depending upon the number of linked moieties and/or functions of the linker, q is generally 1 to 4; m is 1 or more, generally 1 or 2. When more than one facilitating agent and/or mutant analyte binding enzyme is/are present in a conjugate the each agent may be the same or different and each mutant analyte binding enzyme may be the same or different.

25 The conjugates can be produced by any means, including, by chemical conjugation methods and, where both moieties are proteinaceous, as fusion proteins. The conjugates can include a fusion protein portion and a chemically linked portion or any combination thereof.

Any agent, such as a protein or peptide fragment or other moiety that facilitates: i) affinity isolation or purification of the fusion protein; ii) attachment of the fusion protein to a

30 surface; or iii) detection of the fusion protein, is contemplated for use in the conjugate. In one exemplary embodiment, the facilitating agent is a protein binding moiety, such as an epitope tag or an IgG binding protein, a nucleotide binding protein such as a DNA or RNA binding protein, a lipid binding protein, a polysaccharide binding protein, or a metal binding protein. In

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another exemplary embodiment, the facilitating agent is derived from an enzyme, a transport protein, a nutrient or storage protein, a contractile or motile protein, a structural protein, a defense protein, a regulatory protein, or a fluorescent protein.

5   Also provided herein are isolated nucleic acid molecules that contain a sequence of nucleotides encoding the fusion protein. Plasmids containing the molecules, and cells containing the plasmids are also provided. Methods for producing the fusion proteins by culturing the cells containing the plasmids under conditions whereby the fusion protein is expressed by the cell, and recovering the expressed fusion protein are provided.

        Further provided herein are methods for assaying an analyte in a sample using the
10   conjugates. In practicing these methods, the conjugate is contacted with the sample, and interaction between the analyte or an immediate analyte enzymatic conversion product and the conjugate is detected. The presence or amount of the analyte in the sample is then assessed. Prior to the contact between the sample and the conjugate, the conjugate could be isolated or purified through affinity binding between the facilitating agent and an affinity binding moiety.
15   In addition, prior to the contact between the sample and the conjugate, the conjugate can be linked, directly or indirectly, to a surface preferably through affinity binding between the facilitating agent and an affinity binding moiety on the surface, thereby readily permitting solid phase assays to be performed.

        Particular compositions, combinations, kits and articles of manufacture for assaying
20   analytes, preferably small molecule analytes, and methods are described in the sections and subsections that follow.

        High throughput protocols

        The methods and compositions provided herein may be adapted for use in high throughput protocols. In particular, solid supports with a plurality of linked mutant analyte
25   binding enzymes and/or conjugate provided herein may used to screen a plurality of samples. Each of the linked enzymes or conjugates may be the same or different from each other.

BRIEF DESCRIPTION OF THE DRAWINGS

        Fig. 1 depicts Hcy assay using wild type and mutant SAH hydrolase.

        Fig. 2 depicts total plasma Hcy assay procedure with wild type and mutant SAH
30   hydrolase.

        Fig. 3 depicts design and synthesis of fluorescence labeled tracer.

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Fig. 4 depicts selection of mutant SAH hydrolase that lacks catalytic activity but retains substrate binding affinity.

DETAILED DESCRIPTION OF THE INVENTION

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A.   DEFINITIONS

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of ordinary skill in the art to which this invention belongs. All patents, applications, published applications and other publications and sequences from GenBank and other data bases referred to herein are incorporated by reference in their entirety.

As used herein, "analyte" refers to a molecule that can specifically bind to an enzyme, either as a co-enzyme, a co-factor or a substrate.

As used herein, "enzyme" refers to a protein specialized to catalyze or promote a specific metabolic reaction. Generally, enzymes are catalysts, but for purposes herein, such "enzymes" include those that would be modified during a reaction. Since the enzymes are modified to eliminate or substantially eliminate catalytic activity, they will not be so-modified during a reaction.

As used herein, "analyte-binding enzyme" refers to an enzyme that uses the analyte as its co-enzyme, co-factor, or as a substrate. For instance, "Hcy-binding enzyme" refers to an enzyme that uses Hcy as its co-enzyme, co-factor, or its sole or one of its substrates. Examples of Hcy-binding enzymes include SAH hydrolase, cystathionine ß-synthase, methionine synthase, betaine-homocysteine methyltransferase and methioninase. It is intended that analyte-binding enzymes include those conservative amino acid substitutions that do not substantially alter its activity. Suitable conservative substitutions of amino acids are known to those of skill in this art and may be made generally without altering the biological activity of the resulting molecule. Those of skill in this art recognize that, in general, single amino acid substitutions in non-essential regions of a polypeptide do not substantially alter biological

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activity (see, e.g., Watson, et al., Molecular Biology of the Gene, 4th Edition, 1987, The
Bejacmin/Cummings Pub. Co., p. 224).

Such substitutions are preferably made in accordance with those set forth in TABLE 1
as follows:

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TABLE 1

Original residue	Conservative substitution
Ala (A)	Gly; Ser
Arg (R)	Lys
Asn (N)	Gln; His
Cys (C)	Ser
Gln (Q)	Asn
Glu (E)	Asp
Gly (G)	Ala; Pro
His (H)	Asn; Gln
Ile (I)	Leu; Val
Leu (L)	Ile; Val
Lys (K)	Arg; Gln; Glu
Met (M)	Leu; Tyr; Ile
Phe (F)	Met; Leu; Tyr
Ser (S)	Thr
Thr (T)	Ser
Trp (W)	Tyr
Tyr (Y)	Trp; Phe
Val (V)	Ile; Leu

Other substitutions are also permissible and may be determined empirically or in accord with
known conservative substitutions.

As used herein, the "amino acids," which occur in the various amino acid sequences
appearing herein, are identified according to their well-known, three-letter or one-letter
10   abbreviations. The nucleotides, which occur in the various DNA fragments, are designated
with the standard single-letter designations used routinely in the art.

As used herein, "a mutant analyte-binding enzyme" (used interchangeably with
"modified enzyme" and "substrate trapping enzyme" that substantially retains its binding
affinity or has enhanced binding affinity for the analyte or an immediate analyte enzymatic
15   conversion product" refers to a mutant form of an analyte-binding enzyme that retains
sufficient binding affinity for the analyte to be detected in the process or method, particularly
assay, of interest. Typically this is at least about 10%, preferably at least about 50% binding
affinity for the analyte or an immediate analyte enzymatic conversion product, compared to its
wildtype counterpart. Preferably, such mutant analyte-binding enzyme retains 60%, 70%, 80%,
20   90%, 100% binding affinity for the analyte or an immediate analyte enzymatic conversion

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product compared to its wildtype counterpart, or has a higher binding affinity than its wildtype counterpart. Such mutant analyte-binding enzyme is herein referred to as a "substrate trapping enzyme", i.e., a molecule that specifically binds to a selected analyte or target molecule, but does not catalyze conversion thereof.

5   As used herein, "immediate analyte enzymatic conversion product" refers to a product derived from the analyte by catalysis of a single analyte-binding enzyme. For example, the "immediate Hcy enzymatic conversion product" of SAH hydrolase is SAH. The "immediate Hcy enzymatic conversion product" of cystathionine ß-synthase is cystathionine. The "immediate Hcy enzymatic conversion product" of methionine synthase and betaine-

10   homocysteine methyltransferase is methionine.

As used herein, a conjugate refers to the compounds provided herein that include one or more mutant analyte-binding enzymes and one or more facilitating agents. These conjugates include those produced by recombinant means as fusion proteins, those produced by chemical means, such as by chemical coupling, through, for example, coupling to sulfhydryl groups, and

15   those produced by any other method whereby at least one mutant analyte-binding enzyme is linked, directly or indirectly via linker(s) to a facilitating agent.

As used herein, a facilitating agent, is any moiety, such as a protein or effective portion thereof, that promotes or facilitates, for example, preferably:

          i)   affinity isolation or purification of the conjugate;

20   ii)   attachment of the conjugate to a surface; or

          iii)   detection of the conjugate or complexes containing the conjugate.

As used herein the term "assessing" is intended to include quantitative and qualitative determination in the sense of obtaining an absolute value for the amount or concentration of the analyte, e.g., a homocysteine co-substrate, present in the sample, and also of obtaining an

25   index, ratio, percentage, visual or other value indicative of the level of analyte in the sample. Assessment may be direct or indirect and the chemical ʳ̣ ̣ ̣ies actually detected need not of course be the analyte itself but may for example be a derivative thereof or some further substance.

As used herein, "attenuated catalytic activity" refers to a mutant analyte-binding

30   enzyme that retains sufficiently reduced catalytic activity to be useful as a "pseudo-antibody," i.e., a molecule used in place of an antibody in immunoassay formats. The precise reduction in catalytic activity for use in the assays can be empirically determined for each assay. Typically, the enzyme will retain less than about 50% of one of its catalytic activities or less than 50% of

its overall catalytic activities compared to its wildtype counterpart. Preferably, a mutant analyte-binding enzyme retains less than 40%, 30%, 20%, 10%, 1%, 0.1%, or 0.01% of one of its catalytic activities or its overall catalytic activities compared to its wildtype counterpart. More preferably, a mutant analyte-binding enzyme lacks detectable level of one of its catalytic

5   activities or its overall catalytic activities compared to its wildtype counterpart. In instances in which catalytic activity is retained and/or a further reduction thereof is desired, the contacting step can be effected in the presence of a catalysis inhibitor. Such inhibitors, include, but are not limited to, heavy metals, chelators or other agents that bind to a co-factor required for catalysis, but not for binding, and other such agents.

10   As used herein, "macromolecule" refers to a molecule that, without attaching to another molecule, is capable of generating an antibody that specifically binds to the macromolecule.

As used herein, "small molecule" refers to a molecule that, without forming homo-aggregates or without attaching to a macromolecule or adjuvant, is incapable of generating an antibody that specifically binds to the small molecule. Preferably, the small molecule has a

15   molecular weight that is about or less than 10,000 daltons. More preferably, the small molecule has a molecular weight that is about or less than 5,000 dalton.

As used herein, "inorganic molecule" refers to a molecule that does not contain hydrocarbon group(s).

As used herein, "organic molecule" refers to a molecule that contains hydrocarbon

20   group(s).

As used herein, "vitamin" refers to a trace organic substance required in certain biological species. Most vitamins function as components of certain coenzymes.

As used herein, "biomolecule" refers to an organic compound normally present as an essential component of living organisms.

25   As used herein, "lipid" refers to water-insoluble, oily or greasy organic substances that are extractable from cells and tissues by nonpolar solvents, such as chloroform or ether.

As used herein, "homocysteine" (Hcy) refers to a compound with the following molecular formula: $HSCH_2CH_2CH(NH_2)COOH$. Biologically, Hcy is produced by demethylation of methionine and is an intermediate in the biosynthesis of cysteine from

30   methionine. The term "Hcy" encompasses free Hcy (in the reduced form) and conjugated Hcy (in the oxidized form). Hcy can conjugate with proteins, peptides, itself or other thiols through disulfide bond.

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As used herein, "SAH hydrolase" refers to an ubiquitous eukaryotic enzyme, which is also found in some prokaryotes, which catalyzes hydrolysis of SAH to Ado and Hcy. SAH hydrolase also catalyzes the formation of SAH from Ado and Hcy. The co-enzyme of SAH hydrolase is NAD$^+$/NADH. SAH hydrolase has several catalytic activities. In the hydrolytic

5   direction, the first step involves oxidation of the 3'-hydroxyl group of SAH (3'-oxidative activity) by enzyme-bound NAD$^+$ (E-NAD$^+$), followed by ß-elimination of L-Hcy to give 3'-keto-4',5'-didehydro-5'-deoxy-Ado. Michael addition of water to the 5'-position to this tightly bound intermediate (5'-hydrolytic activity) affords 3'-keto-Ado, which is then reduced by enzyme-bound NADH (E-NADH) to Ado (3'-reduction activity). It is intended to encompass

10   SAH hydrolase with conservative amino acid substitutions that do not substantially alter its activity.

As used herein, "SAH hydrolase catalysis inhibitor" refers to an agent that inhibits one or all of SAH hydrolase catalytic activities, e.g., 3'-oxidative activity, 5'-hydrolytic activity, or 3'-reduction activity, while not affecting SAH hydrolase's binding affinity for Hcy and/or SAH.

15   As used herein, "cystathionine ß-synthase" refers to an enzyme that irreversibly catalyzes the formation of cystathionine from Hcy and serine. The co-enzyme of cystathionine ß-synthase is pyridoxal 5'-phosphate. It is intended to encompass cystathionine ß-synthase with conservative amino acid substitutions that do not substantially alter its activity.

As used herein, "methionine synthase" refers to an enzyme that irreversibly catalyzes

20   the formation of methionine from Hcy and 5-methyltetrahydrofolate (5-CH$_3$-THF). The co-enzyme of cystathionine ß-synthase is vitamin B$_{12}$. It is intended to encompass methionine synthase with conservative amino acid substitutions that do not substantially alter its activity.

As used herein, "betaine-homocysteine methyltransferase" refers to an enzyme that irreversibly catalyzes the formation of methionine and dimethyl-glycine from Hcy and betaine.

25   It is intended to encompass betaine-homocysteine methyltransferase with conservative amino acid substitutions that do not substantially alter its activity.

As used herein, "methioninase" refers to an enzyme that catalyzes α, ß- and α, Γ-eliminations from S-substituted amino acids and also catalyzes a variety of ß- and Γ-exchange reactions, according to the following equations: RSCH$_2$CH(NH$_2$)COOH+R'SH in

30   equilibrium with R'SCH2CH(NH2)COOH+RSH (ß-exchange) and RSCH$_2$CH$_2$CH(NH$_2$)COOH + R'SH in equilibrium with R'SCH$_2$CH$_2$CH(NH$_2$)COOH + RSH (Γ-exchange), where R'SH represents an alkanethiol or a substituted thiol (Ito, et al., J. Biochem., (Tokyo) 80(6):1327-34 (1976)). In particular, R and R' independently are selected preferably from alkyl, aryl, alkynyl,

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cycloalkly, heteroaryl, alkenyl, amino acids, proteins and other suitable moieties or mixtures thereof. R and R' typically contain less than about 50 atoms, are substituted or unsubstituted, the carbon chains can be straight or branched or cyclized, heteroatoms include S, N, O; the aryl and heteroaryl or other cyclic groups can include one ring or two or more fused rings, each ring

5   preferably containing from 3 to 7, more preferably 4 to 6, members.

As used herein, "adenosine deaminase" refers to an enzyme that catalyzes the deamination of adenosine to form inosine. It is intended to encompass adenosine deaminase with conservative amino acid substitutions that do not substantially alter its activity.

As used herein, "purine nucleoside phosphorylase" refers to an enzyme that catalyzes

10   the formation of hypoxanthine and D-ribose from inosine and water. It is intended to encompass purine nucleoside phosphorylase with conservative amino acid substitutions that do not substantially alter its activity.

As used herein, "xanthine oxidase" refers to an enzyme that catalyzes the conversion of hypoxanthine to uric acid via xanthine. It is intended to encompass xanthine oxidase with

15   conservative amino acid substitutions that do not substantially alter its activity.

As used herein, "folate species" refers to folate or folic acid, which is chemically N-[4-[[2-amino-1,4-dihydro-4-oxo-6-pteridinyl)methyl]amino]benzxoyl]-L-glutamic acid, or a derivative thereof. Examples of folate derivatives include, but are not limited to, dihydrofolate, tetrahydrofolate, 5,-methyl-tetrahydrofolate and 5,10-methylene

20   tetrahydrofolate.

As used herein, "tetrahydrofolate methyltransferase" refers to an enzyme that catalyzes the formation of 5,10-methylene tetrahydrofolate and glycine from tetrahydrofolate and serine. It is intended to encompass tetrahydrofolate methyltransferase with conservative amino acid substitutions that do not substantially alter its activity.

25   As used herein, "methylenetetrahydrofolate reductase" refers to an enzyme that catalyzes the formation of 5,-methyl-tetrahydrofolate from 5,10-methylene tetrahydrofolate. It is intended to encompass methylenetetrahydrofolate reductase with conservative amino acid substitutions that do not substantially alter its activity.

As used herein, "folypolyglutamate synthase" refers to an enzyme that catalyzes the

30   formation of 5,10-methylenetetrahydrofolate-diglutamate derivative, ADP and Pi from 5,10-methylenetetrahydrofolate, L-glutamate and ATP. The cofactor of folypolyglutamate synthase is Mg^{2+}. It is intended to encompass folypolyglutamate synthase with conservative amino acid substitutions that do not substantially alter its activity.

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As used herein, "dihydrofolate reductase" refers to an enzyme that catalyzes the formation of tetrahydrofolate and $NADP^+$ from dihydrofolate, NADPH and H^+. It is intended to encompass dihydrofolate reductase with conservative amino acid substitutions that do not substantially alter its activity.

5   As used herein, "thymidylate synthase" refers to an enzyme that catalyzes the formation of dihydrofolate and dTMP from 5,10-methylenetetrahydrofolate and dUMP. It is intended to encompass thymidylate synthase with conservative amino acid substitutions that do not substantially alter its activity.

As used herein, "cholesterol esterase" refers to an enzyme that catalyzes the formation
10   of cholesterol and fatty acids from cholesterolester and H_2O. It is intended to encompass cholesterol esterase with conservative amino acid substitutions that do not substantially alter its activity.

As used herein, "cholesterol oxidase" refers to an enzyme that catalyzes the formation of cholesterol-4-en-3-one and H_2O_2 from cholesterol and O_2. It is intended to encompass
15   cholesterol oxidase with conservative amino acid substitutions that do not substantially alter its activity.

As used herein, "calcineurin (also called phosphoprotein phosphatase 2B or PP2B)" refers to a Ca^{2+}/calmodulin-dependent protein phosphatase that is an element of many intracellular signaling pathways including T cell activation. In T cells, calcineurin participates
20   in regulation of IL-2 expression following T cell stimulation. Nuclear factor of activated T cells ($NFAT_p$) has been shown to be a substrate for calcineurin phosphatase activity. Following T cell stimulation, calcineurin-mediated $NFAT_p$ dephosphorylation allows translocation of $NFAT_p$ from the cytoplasm to the nucleus where $NFAT_p$ interacts with Fos and Jun to induce expression of the IL-2 gene. It is intended to encompass calcineurin with conservative amino
25   acid substitutions that do not substantially alter its activity.

As used herein, "catechol O-methyltransferase (COMT)" refers to an enzyme that catalyzes the transfer of the methyl group of S-adenosyl-L-methionine (AdoMet) to one of the hydroxyl groups of a catechol substrate in the presence of Mg^{2+}. The physiological substrates of COMT include dopa, catecholamines (e.g., dopamine, noradrenaline, adrenaline), their
30   hydroxylated metabolites, catechol estrogens and ascorbic acid. COMT is mainly a cellular enzyme. In vertebrates, the COMT protein appears mostly in soluble form and a minor fraction is in a membrane-bound form. It is intended to encompass catechol O-methyltransferase with conservative amino acid substitutions that do not substantially alter its activity.

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As used herein, "creatinine amidohydrolase" refers to an enzyme that catalyses the following reaction:

Creatinine + H$_2$O <---> Creatine.

It is intended to encompass creatinine amidohydrolase with conservative amino acid

5   substitutions that do not substantially alter its activity.

As used herein, "cyclophilin" refers to an enzyme that: 1) has cis-trans peptidyl-prolyl isomerase (PPIase) activity; 2) binds drug cyclosporin A (CsA); and 3) inhibits calcineurin in the presence of CsA. It is intended to encompass cyclophilin with conservative amino acid substitutions that do not substantially alter its activity.

10   As used herein, "dihydroorotate dehydrogenase" refers to an enzyme that catalyzes the conversion of L-dihydroorotate to orotate in the presence of O$_2$. It is intended to encompass dihydroorotate dehydrogenase with conservative amino acid substitutions that do not substantially alter its activity.

As used herein, "dopamine-ß-hydroxylase" refers to an enzyme that hydroxylates

15   dopamine to norepinephrine in the presence of oxygen and ascorbic acid. It is intended to encompass dopamine-ß-hydroxylase with conservative amino acid substitutions that do not substantially alter its activity.

As used herein, "hyaluronidase" refers to the class of enzymes that act on the disaccharide unit of D-glucuronic acid and N-acetyl-D-glucosamine. Such enzymes mediate

20   the hydrolysis of polymers of repeating disaccharides comprising D-glucuronic acid and N-acetyl-D-glucosamine. One example of such polymer is hyaluronic acid. Hyaluronidase catalyzes the release of reducing groups of N-acetylglucosamine from hyaluronic acid. It is intended to encompass hyaluronidase with conservative amino acid substitutions that do not substantially alter its activity.

25   As used herein, "hydroxymethylglutaryl-CoA reductase (HMG-CoA reductase)" refers to an enzyme that catalyzes the conversion of 3-hydroxy-3-methylglutaryl-CoA to mevalonate in a reaction requiring NADPH as the co-enzyme. It is intended to encompass HMG-CoA reductase with conservative amino acid substitutions that do not substantially alter its activity.

As used herein, "hydroxysteroid dehydrogenase" refers to a family of enzymes which

30   play a pivotal role in the regulation of steroid hormone action. These enzymes catalyze the interconversion of secondary alcohols to ketones in a positional and stereospecific manner on the steroid nucleus and side chain. They require nicotinamide dinucleotide (phosphate) NADP$^+$ as cofactor. For example, 3α-hydroxysteroid dehydrogenase catalyzes the reduction

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5α-dihydrotestosterone to 5α-androstan-3α,17β-diol. It is intended to encompass
hydroxysteroid dehydrogenase with conservative amino acid substitutions that do not
substantially alter its activity.

As used herein, "inosine-5'-monophosphate dehydrogenase (IMPDH)" refers to an
5 enzyme that is involved in the *de novo* synthesis of guanosine nucleotides. IMPDH catalyzes
the NAD⁺-dependent oxidation of inosine-5'-monophosphate (IMP) to xanthosine-5'-
monophosphate (XMP). IMPDH is ubiquitous in eukaryotes, bacteria and protozoa.
Regardless of species, the enzyme follows an ordered Bi--Bi reaction sequence of substrate and
cofactor binding and product release. First, IMP binds to IMPDH. This is followed by the
10 binding of the cofactor NAD⁺. The reduced cofactor, NADH, is then released from the
complex, followed by the product, XMP. It is intended to encompass IMPDH with
conservative amino acid substitutions that do not substantially alter its activity.

As used herein, "monoamine oxidase" refers to an enzyme that catalyzes the oxidative
deamination of a wide variety of dietary amines and neurotransmitters such as dopamine,
15 norepinephrine, and serotonin. It is an integral protein of the outer mitochondrial membrane
and is present in all types of cells. Two isoenzymic forms (Types A and B) have been
identified. It is intended to encompass monoamine oxidase with conservative amino acid
substitutions that do not substantially alter its activity.

As used herein, "procainamide N-acetyltransferase" refers to an enzyme that catalyzes
20 the transfer of the acetyl moiety of acetyl CoA to an acceptor amine such as procainamide. It is
intended to encompass serotonin N-acetyltransferase with conservative amino acid
substitutions that do not substantially alter its activity.

As used herein, "serotonin N-acetyltransferase (AANAT)" refers to an enzyme that
catalyzes the conversion of serotonin to N-acetylserotonin in a reaction requiring acetyl
25 coenzyme A (AcCoA). It is intended to encompass serotonin N-acetyltransferase with
conservative amino acid substitutions that do not substantially alter its activity.

As used herein, "bile acid" refers to acidic sterols synthesized from cholesterol in the
liver. Following synthesis, the bile acids are secreted into bile and enter the lumen of the small
intestine, where they facilitate absorption of fat-soluble vitamins and cholesterol. In humans,
30 the most abundant bile acid is cholic acid.

As used herein, "bile salt" refers to salt of bile acid. The major human bile salts are
sodium glycocholate and sodium taurocholate.

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As used herein, "3-α-hydroxy steroid dehydrogenase" refers to an enzyme that catalyzes the 3-oxo-bile-acid, H^+ and NADH from 3-α-hydroxy-bile-acid and NAD^+. It is intended to encompass 3-α-hydroxy steroid dehydrogenase with conservative amino acid substitutions that do not substantially alter its activity.

5   As used herein, "glucose isomerase" refers to an enzyme that catalyzes the reversible conversion between D-glucose and D-fructose. It is intended to encompass glucose isomerase with conservative amino acid substitutions that do not substantially alter its activity.

As used herein, "hexokinase or glucokinase" refers to an enzyme that catalyzes the formation of D-glucose 6-phosphate and ADP from α-D-glucose and ATP. The cofactor of

10   hexokinase or glucokinase is Mg^{2+}. It is intended to encompass hexokinase or glucokinase with conservative amino acid substitutions that do not substantially alter its activity.

As used herein, "glucose oxidase" refers to an enzyme that catalyzes the formation of gluconic acid and H_2O_2 from glucose, H_2O and O_2. It is intended to encompass glucose oxidase with conservative amino acid substitutions that do not substantially alter its activity.

15   As used herein, "alcohol dehydrogenase" refers to an enzyme that catalyzes the formation of acetaldehyde, NADH and H^+ from ethanol and NAD^+. The cofactor of alcohol dehydrogenase is Zn^{2+}. It is intended to encompass alcohol dehydrogenase with conservative amino acid substitutions that do not substantially alter its activity.

As used herein, "urate oxidase or uricase" refers to an enzyme that catalyzes the

20   formation of allantoin and CO_2 from uric acid, O_2 and H_2O. The cofactor of urate oxidase or uricase is copper. It is intended to encompass urate oxidase or uricase with conservative amino acid substitutions that do not substantially alter its activity.

As used herein, "serum" refers to the fluid portion of the blood obtained after removal of the fibrin clot and blood cells, distinguished from the plasma in circulating blood.

25   As used herein, "plasma" refers to the fluid, noncellular portion of the blood, distinguished from the serum obtained after coagulation.

As used herein, "substantially pure" means sufficiently homogeneous to appear free of readily detectable impurities as determined by standard methods of analysis, such as thin layer chromatography (TLC), gel electrophoresis and high performance liquid chromatography

30   (HPLC), used by those of skill in the art to assess such purity, or sufficiently pure such that further purification would not detectably alter the physical and chemical properties, such as enzymatic and biological activities, of the substance. Methods for purification of the compounds to produce substantially chemically pure compounds are known to those of skill in

the art. A substantially chemically pure compound may, however, be a mixture of stereoisomers or isomers. In such instances, further purification might increase the specific activity of the compound.

As used herein, "biological activity" refers to the in vivo activities of a compound or physiological responses that result upon in vivo administration of a compound, composition or other mixture. Biological activity, thus, encompasses therapeutic effects and pharmaceutical activity of such compounds, compositions and mixtures. Biological activities may be observed in vitro systems designed to test or use such activities. Thus, for purposes herein the biological activity of a luciferase is its oxygenase activity whereby, upon oxidation of a substrate, light is produced.

As used herein, a "receptor" refers to a molecule that has an affinity for a given ligand. Receptors may be naturally-occurring or synthetic molecules. Receptors may also be referred to in the art as anti-ligands. As used herein, the receptor and anti-ligand are interchangeable. Receptors can be used in their unaltered state or as aggregates with other species. Receptors may be attached, covalently or noncovalently, or in physical contact with, to a binding member, either directly or indirectly via a specific binding substance or linker. Examples of receptors, include, but are not limited to: antibodies, cell membrane receptors surface receptors and internalizing receptors, monoclonal antibodies and antisera reactive with specific antigenic determinants [such as on viruses, cells, or other materials], drugs, polynucleotides, nucleic acids, peptides, cofactors, lectins, sugars, polysaccharides, cells, cellular membranes, and organelles.

Examples of receptors and applications using such receptors, include but are not restricted to:

a) enzymes: specific transport proteins or enzymes essential to survival of microorganisms, which could serve as targets for antibiotic [ligand] selection;

b) antibodies: identification of a ligand-binding site on the antibody molecule that combines with the epitope of an antigen of interest may be investigated; determination of a sequence that mimics an antigenic epitope may lead to the development of vaccines of which the immunogen is based on one or more of such sequences or lead to the development of related diagnostic agents or compounds useful in therapeutic treatments such as for auto-immune diseases

c) nucleic acids: identification of ligand, such as protein or RNA, binding sites;

d) catalytic polypeptides: polymers, preferably polypeptides, that are capable of
promoting a chemical reaction involving the conversion of one or more reactants to one or
more products; such polypeptides generally include a binding site specific for at least one
reactant or reaction intermediate and an active functionality proximate to the binding site, in
5   which the functionality is capable of chemically modifying the bound reactant [see, *e.g.*, U.S.
Patent No. 5,215,899];

e) hormone receptors: determination of the ligands that bind with high affinity to a
receptor is useful in the development of hormone replacement therapies; for example,
identification of ligands that bind to such receptors may lead to the development of drugs to
10   control blood pressure; and

f) opiate receptors: determination of ligands that bind to the opiate receptors in the brain
is useful in the development of less-addictive replacements for morphine and related drugs.

As used herein, "antibody" includes antibody fragments, such as Fab fragments, which
are composed of a light chain and the variable region of a heavy chain.

15   As used herein, "humanized antibodies" refer to antibodies that are modified to include
"human" sequences of amino acids so that administration to a human will not provoke an
immune response. Methods for preparation of such antibodies are known. For example, the
hybridoma that expresses the monoclonal antibody is altered by recombinant DNA techniques
to express an antibody in which the amino acid composition of the non-variable regions is
20   based on human antibodies. Computer programs have been designed to identify such regions.

As used herein, "production by recombinant means" refers to production methods that
use recombinant nucleic acid methods that rely on well known methods of molecular biology
for expressing proteins encoded by cloned nucleic acids.

As used herein, "substantially identical" to a product means sufficiently similar so that
25   the property of interest is sufficiently unchanged so that the substantially identical product can
be used in place of the product.

As used herein, "equivalent," when referring to two sequences of nucleic acids means
that the two sequences in question encode the same sequence of amino acids or equivalent
proteins. It also encompasses those that hybridize under conditions of moderate, preferably
30   high stringency, whereby the encoded protein retains desired properties.

As used herein, when "equivalent" is used in referring to two proteins or peptides, it
means that the two proteins or peptides have substantially the same amino acid sequence with

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only conservative amino acid substitutions [see, e.g., Table 1, above] that do not substantially alter the activity or function of the protein or peptide.

When "equivalent" refers to a property, the property does not need to be present to the same extent [e.g., two peptides can exhibit different rates of the same type of enzymatic

5   activity], but the activities are preferably substantially the same. "Complementary," when referring to two nucleic acid molecules, means that the two sequences of nucleotides are capable of hybridizing, preferably with less than 25%, more preferably with less than 15%, even more preferably with less than 5%, most preferably with no mismatches between opposed nucleotides. Preferably the two molecules will hybridize under conditions of high stringency.

10   As used herein: "stringency of hybridization" in determining percentage mismatch is as follows:

       1) high stringency: 0.1 x SSPE, 0.1% SDS, 65°C;

       2) medium stringency: 0.2 x SSPE, 0.1% SDS, 50°C (also referred to as moderate stringency); and

15   3) low stringency: 1.0 x SSPE, 0.1% SDS, 50°C.

It is understood that equivalent stringencies may be achieved using alternative buffers, salts and temperatures.

The term "substantially" identical or homologous or similar varies with the context as understood by those skilled in the relevant art and generally means at least 70%, preferably

20   means at least 80%, more preferably at least 90%, and most preferably at least 95% identity.

As used herein, a "composition" refers to a any mixture of two or more products or compounds. It may be a solution, a suspension, liquid, powder, a paste, aqueous, non-aqueous or any combination thereof.

As used herein, a "combination" refers to any association between two or among more

25   items.

As used herein, "fluid" refers to any composition that can flow. Fluids thus encompass compositions that are in the form of semi-solids, pastes, solutions, aqueous mixtures, gels, lotions, creams and other such compositions.

As used herein, "vector (or plasmid)" refers to discrete elements that are used to

30   introduce heterologous DNA into cells for either expression or replication thereof. Selection and use of such vehicles are well known within the skill of the artisan. An expression vector includes vectors capable of expressing DNA's that are operatively linked with regulatory

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sequences, such as promoter regions, that are capable of effecting expression of such DNA fragments. Thus, an expression vector refers to a recombinant DNA or RNA construct, such as a plasmid, a phage, recombinant virus or other vector that, upon introduction into an appropriate host cell, results in expression of the cloned DNA. Appropriate expression vectors

5   are well known to those of skill in the art and include those that are replicable in eukaryotic cells and/or prokaryotic cells and those that remain episomal or those which integrate into the host cell genome.

As used herein, "a promoter region or promoter element" refers to a segment of DNA or RNA that controls transcription of the DNA or RNA to which it is operatively linked. The

10   promoter region includes specific sequences that are sufficient for RNA polymerase recognition, binding and transcription initiation. This portion of the promoter region is referred to as the promoter. In addition, the promoter region includes sequences that modulate this recognition, binding and transcription initiation activity of RNA polymerase. These sequences may be *cis* acting or may be responsive to *trans* acting factors. Promoters, depending upon the

15   nature of the regulation, may be constitutive or regulated. Exemplary promoters contemplated for use in prokaryotes include the bacteriophage T7 and T3 promoters, and the like.

As used herein, "operatively linked or operationally associated" refers to the functional relationship of DNA with regulatory and effector sequences of nucleotides, such as promoters, enhancers, transcriptional and translational stop sites, and other signal sequences. For example,

20   operative linkage of DNA to a promoter refers to the physical and functional relationship between the DNA and the promoter such that the transcription of such DNA is initiated from the promoter by an RNA polymerase that specifically recognizes, binds to and transcribes the DNA. In order to optimize expression and/or *in vitro* transcription, it may be necessary to remove, add or alter 5' untranslated portions of the clones to eliminate extra, potential

25   inappropriate alternative translation initiation (*i.e.*, start) codons or other sequences that may interfere with or reduce expression, either at the level of transcription or translation. Alternatively, consensus ribosome binding sites (see, *e.g.*, Kozak, *J. Biol. Chem.*, 266:19867-19870 (1991)) can be inserted immediately 5' of the start codon and may enhance expression. The desirability of (or need for) such modification may be empirically determined.

30   As used herein, "sample" refers to anything which may contain an analyte for which an analyte assay is desired. The sample may be a biological sample, such as a biological fluid or a biological tissue. Examples of biological fluids include urine, blood, plasma, serum, saliva, semen, stool, sputum, cerebral spinal fluid, tears, mucus, amniotic fluid or the like. Biological

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tissues are aggregates of cells, usually of a particular kind together with their intercellular substance that form one of the structural materials of a human, animal, plant, bacterial, fungal or viral structure, including connective, epithelium, muscle and nerve tissues. Examples of biological tissues also include organs, tumors, lymph nodes, arteries and individual cell(s).

5   As used herein, the abbreviations for any protective groups, amino acids and other compounds, are, unless indicated otherwise, in accord with their common usage, recognized abbreviations, or the IUPAC-IUB Commission on Biochemical Nomenclature (see, (1972) Biochem. 11:1726).

As used herein, "protein binding sequence" refers to a protein or peptide sequence that 10 is capable of specific binding to other protein or peptide sequences generally, to a set of protein or peptide sequences or to a particular protein or peptide sequence.

As used herein, "epitope tag" refers to a short stretch of amino acid residues corresponding to an epitope to facilitate subsequent biochemical and immunological analysis of the "epitope tagged" protein or peptide. "Epitope tagging" is achieved by appending the 15 sequence of the "epitope tag" to the protein-encoding sequence in an appropriate expression vector. "Epitope tagged" proteins can be affinity purified using highly specific antibodies raised against the tags.

As used herein, "Protein A or Protein G" refers to proteins that can bind to Fc region of most IgG isotypes. Protein A or Protein G are typically found in the cell wall of some strains 20 of *staphylococci*. It is intended to encompass Protein A or Protein G with conservative amino acid substitutions that do not substantially alter its activity.

As used herein, "nucleotide binding sequence" refers to a protein or peptide sequence that is capable of specific binding to nucleotide sequences generally, to a set of nucleotide sequences or to a particular nucleotide sequence.

25   As used herein, "A-form DNA" refers to a DNA structure wherein the presence of the 2' hydroxyl group prevents adoption of the B-form. The A-form DNA structure is very close to the conformation of double-stranded RNA. Hybrid duplexes with one strand of DNA and one strand of RNA also lie in the A-form.

As used herein, "B-form DNA" refers to a DNA structure that follows the Watson and 30 Crick model and represents the general structure of DNA. The DNA in living cells exist in the B-form.

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As used herein, "Z-form DNA" refers to a DNA structure that follows the left-handed helix. The Z-form double helix occurs in polymers that have a sequence of alternating purines and pyrimidines.

As used herein, "replication" refers to a process of DNA-dependent DNA synthesis wherein the DNA molecule is duplicated to give identical copies.

As used herein, "transcription" refers to a process of DNA-dependent RNA synthesis.

As used herein, "DNA repair" refers to a process wherein the sites of mutations in DNA are recognized by special nuclease that excise the damaged region from DNA; and then further enzymes synthesize a replacement sequence so that the original DNA sequence is preserved.

As used herein, "recombination" refers to a reaction between homologous sequences of DNA. The critical feature is that the enzymes responsible for recombination can use any pair of homologous sequences as substrates, although some types of sequences may be favored over others. Recombination allows favorable or unfavorable mutations to be separated and tested as individual units in new assortments.

As used herein, "DNA structure maintenance" refers to DNA sequences, through binding to proteins, that maintain the DNA molecule in particular structures such as chromatids, chromatins or chromosomes.

As used herein, "DNA polymerase" refers to an enzyme that synthesizes DNA using a DNA as the template. It is intended to encompass DNA polymerase with conservative amino acid substitutions that do not substantially alter its activity.

As used herein, "DNA-dependent RNA polymerase" or "transcriptase" refers to an enzyme that synthesizes RNA using a DNA as the template. It is intended to encompass DNA-dependent RNA polymerase with conservative amino acid substitutions that do not substantially alter its activity.

As used herein, "DNAase" refers to an enzyme that attacks bonds in DNA. It is intended to encompass DNAase with conservative amino acid substitutions that do not substantially alter its activity.

As used herein, "DNA ligase" refers to an enzyme that catalyses the formation of a phosphodiester bond to link two adjacent bases separated by a nick in one strand of double helix of DNA. It is intended to encompass DNA ligase with conservative amino acid substitutions that do not substantially alter its activity.

As used herein, "DNA topoisomerase" refers to an enzyme that can change the linking number of DNA. It is intended to encompass DNA topoisomerase with conservative amino acid substitutions that do not substantially alter its activity.

As used herein, "DNA transposase" refers to an enzyme that is involved in insertion of a
5   transposon at a new site. It is intended to encompass DNA transposase with conservative amino acid substitutions that do not substantially alter its activity.

As used herein, "Transposon" refers to a DNA sequence that is able to replicate and insert one copy at a new location in the genome.

As used herein, "DNA kinase" refers to an enzyme that phosphorylates DNA. It is
10 intended to encompass DNA kinase with conservative amino acid substitutions that do not substantially alter its activity.

As used herein, "restriction enzyme" refers to an enzyme that recognizes specific short sequences of DNA and cleaves the duplex at the recognition site or other site. It is intended to encompass a restriction enzyme with conservative amino acid substitutions that do not
15 substantially alter its activity.

As used herein, "rRNA" or "ribosomal RNA" refers to the RNA components of the ribosome, a compact ribonucleoprotein particle that assembles amino acids into proteins.

As used herein, "mRNA" or "messenger RNA" refers to the RNA molecule that bears the same sequence of the DNA coding strand and is used as the template in protein synthesis.
20 As used herein, "tRNA" or "transfer RNA" refers to the RNA molecule that carries amino acids to the ribosome for protein synthesis.

As used herein, "reverse transcription" refers to the RNA-dependent DNA synthesis.

As used herein, "RNA splicing" refers to the removal of introns and joining of exons in RNA so that introns are spliced out and exons are spliced together.
25 As used herein, "RNA-dependent DNA polymerase" or "reverse transcriptase" refers to an enzyme that synthesizes DNA using a RNA as the template. It is intended to encompass a RNA-dependent DNA polymerase with conservative amino acid substitutions that do not substantially alter its activity.

As used herein, "RNA-dependent RNA polymerase" refers to an enzyme that
30 synthesizes RNA using a RNA as the template. It is intended to encompass a RNA-dependent RNA polymerase with conservative amino acid substitutions that do not substantially alter its activity.

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As used herein, "RNA ligase" refers to an enzyme that catalyses the formation of a phosphodiester bond to link two adjacent bases separated by a nick in one strand of RNA. It is intended to encompass a RNA ligase with conservative amino acid substitutions that do not substantially alter its activity.

5   As used herein, "RNA maturase" refers to an enzyme that catalyses the removal of intron in the RNA splicing. It is intended to encompass a RNA maturase with conservative amino acid substitutions that do not substantially alter its activity.

As used herein, "lipid binding sequence" refers to a protein or peptide sequence that is capable of specific binding to lipids generally, to a set of lipids or to a particular lipid.

10   As used herein, "C2 motif" refers to a protein domain that has the similar binding affinity as the C2 domain of approximately 130 residues in length originally identified in the Ca^{2+}-dependent isoforms of protein kinase C (Nalefski and Falke, *Protein Sci.*, 5(12):2375-90 (1996)). Single and multiple copies of C2 domains have been identified in a number of eukaryotic signaling proteins that interact with cellular membranes and mediate a broad array

15   of critical intracellular processes, including membrane trafficking, the generation of lipid-second messengers, activation of GTPases, and the control of protein phosphorylation. As a group, C2 domains display the remarkable property of binding a variety of different ligands and substrates, including Ca^{2+}, phospholipids, inositol polyphosphates, and intracellular proteins. C2 domain exists in two topologies: the fold of the original synaptotagmin C2A domain as

20   "topology I," while that of the phosphoinositide-specific phospholipase C-δ1 domain as "topology II." Each of these structures forms an eight-stranded anti-parallel β-sandwich including a pair of four-stranded ß-sheets, with a slight difference in their ß-strand connection.

As used herein, "amphipathic α-helix motif" refers to an α helix with opposing polar and nonpolar faces oriented along its long axis (Segrest, *et al.*, *Adv. Protein Chem.*, 45:303-69

25   (1994)).

As used herein, "polysaccharide binding sequence" refers to a protein or peptide sequence that is capable of specific binding to polysaccharides generally, to a set of polysaccharides or to a particular polysaccharide.

As used herein, "metal binding sequence" refers to a protein or peptide sequence that is

30   capable of specific binding to metal ions generally, to a set of metal ions or to a particular metal ion.

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As used herein, "transport protein" refers to a protein that carries specific molecules or ions from one organ to another. Non-limiting examples of transport proteins include hemoglobin, serum albumin, myoglobin and ß1-lipoprotein.

As used herein, "nutrient or storage protein" refers to a protein that is used by the cell as the nutrient source or storage form for such nutrient. Non-limiting examples of nutrient or storage proteins include gliadin, ovalbumin, casein, and ferritin.

As used herein, "contractile or motile protein" refers to a protein that endows cells and organisms with the ability to contract, to change shape, or to move about. Non-limiting examples of contractile or motile proteins include actin, myosin, tubulin and dynein.

As used herein, "structural protein" refers to a protein that serves as supporting filaments, cables, or sheets to give biological structures strength or protection. Non-limiting examples of structural proteins include keratin, fibroin, collagen, elastin and proteoglycans.

As used herein, "defense protein" refers to a protein that defends organisms against invasion by other species or protect them from injury. Non-limiting examples of defense proteins include antibodies, fibrinogen, thrombin, botulinus toxin, diphtheria toxin, snake venoms and ricin.

As used herein, "regulatory protein" refers to a protein that helps regulate cellular or physiological activity. Non-limiting examples of regulatory proteins include insulin, growth hormones, corticotropin and repressors.

As used herein, "luminescence" refers to the detectable EM radiation, generally, UV, IR or visible EM radiation that is produced when the excited product of an exergic chemical process reverts to its ground state with the emission of light. Chemiluminescence is luminescence that results from a chemical reaction. Bioluminescence is chemiluminescence that results from a chemical reaction using biological molecules or synthetic versions or analogs thereof as substrates and/or enzymes.

As used herein, "bioluminescence," which is a type of chemiluminescence, refers to the emission of light by biological molecules, particularly proteins. The essential condition for bioluminescence is molecular oxygen, either bound or free in the presence of an oxygenase, a luciferase, which acts on a substrate, a luciferin. Bioluminescence is generated by an enzyme or other protein (luciferase) that is an oxygenase that acts on a substrate luciferin (a bioluminescence substrate) in the presence of molecular oxygen and transforms the substrate to an excited state, which upon return to a lower energy level releases the energy in the form of light.

As used herein, the substrates and enzymes for producing bioluminescence are generically referred to as luciferin and luciferase, respectively. When reference is made to a particular species thereof, for clarity, each generic term is used with the name of the organism from which it derives, for example, bacterial luciferin or firefly luciferase.

5   As used herein, "luciferase" refers to oxygenases that catalyze a light emitting reaction. For instance, bacterial luciferases catalyze the oxidation of flavin mononucleotide [FMN] and aliphatic aldehydes, which reaction produces light. Another class of luciferases, found among marine arthropods, catalyzes the oxidation of *Cypridina* [*Vargula*] luciferin, and another class of luciferases catalyzes the oxidation of *Coleoptera* luciferin.

10   Thus, luciferase refers to an enzyme or photoprotein that catalyzes a bioluminescent reaction [a reaction that produces bioluminescence]. The luciferases, such as firefly and *Renilla* luciferases, that are enzymes which act catalytically and are unchanged during the bioluminescence generating reaction. The luciferase photoproteins, such as the aequorin photoprotein to which luciferin is non-covalently bound, are changed, such as by release of the

15   luciferin, during bioluminescence generating reaction. The luciferase is a protein that occurs naturally in an organism or a variant or mutant thereof, such as a variant produced by mutagenesis that has one or more properties, such as thermal stability, that differ from the naturally-occurring protein. Luciferases and modified mutant or variant forms thereof are well known. For purposes herein, reference to luciferase refers to either the photoproteins or

20   luciferases.

As used herein, "peroxidase" refers to an enzyme that catalyses a host of reactions in which hydrogen peroxide is a specific oxidizing agent and a wide range of substrates act as electron donors. It is intended to encompass a peroxidase with conservative amino acid substitutions that do not substantially alter its activity. Peroxidases are widely distributed in

25   nature and are produced by a wide variety of plant species. The chief commercially available peroxidase is horseradish peroxidase.

As used herein, "urease" refers to an enzyme that catalyses decomposition of urea to form ammonia and carbon dioxide. It is intended to encompass an urease with conservative amino acid substitutions that do not substantially alter its activity. Urease is widely found in

30   plants, animals and microorganisms.

As used herein, "alkaline phosphatases" refers to a family of functionally related enzymes named after the tissues in which they predominately appear. Alkaline phosphatases carry out hydrolase/transferase reactions on phosphate-containing substrates at a high pH

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optimum. It is intended to encompass alkaline phosphatases with conservative amino acid

substitutions that do not substantially alter its activity.

As used herein, "glutathione S-transferase" refers to a ubiquitous family of enzymes

with dual substrate specificities that perform important biochemical functions of xenobiotic

5   biotransformation and detoxification, drug metabolism, and protection of tissues against

peroxidative damage. The basic reaction catalyzed by glutathione S-transferase is the

conjugation of an electrophile with reduced glutathione (GSH) and results in either activation

or deactivation/detoxification of the chemical. It is intended to encompass a glutathione S-

transferase with conservative amino acid substitutions that do not substantially alter its activity.

10   As used herein, high-throughput screening (HTS) refers to processes that test a large

number of samples, such as samples of diverse chemical structures against disease targets to

identify "hits" (see, e.g., Broach, et al., High throughput screening for drug discovery, Nature,

384:14-16 (1996); Janzen, et al., High throughput screening as a discovery tool in the

pharmaceutical industry, Lab Robotics Automation: 8261-265 (1996); Fernandes, P.B., Letter

15   from the society president, J. Biomol. Screening, 2:1 (1997); Burbaum, et al., New technologies

for high-throughput screening, Curr. Opin. Chem. Biol., 1:72-78 (1997)]. HTS operations are

highly automated and computerized to handle sample preparation, assay procedures and the

subsequent processing of large volumes of data.

As used herein, "disease or disorder" refers to a pathological condition in an organism

20   resulting from, e.g., infection or genetic defect, and characterized by identifiable symptoms.

As used herein, "infection" refers to invasion of the body of a multi-cellular organism

with organisms that have the potential to cause disease.

As used herein, "infectious organism" refers to an organism that is capable to cause

infection of a multi-cellular organism. Most infectious organisms are microorganisms such as

25   viruses, bacteria and fungi.

For clarity of disclosure, and not by way of limitation, the detailed description is

divided into the subsections that follow.

B.   METHODS FOR ASSAYING ANALYTES

Provided herein are methods for assaying an analyte in a sample. Any assays that

30   employ an antibody as a reagent can be modified as described herein by replacing the antibody

with an enzyme that has been modified such that it retains the ability to bind to an analyte of

interest but has substantially reduced catalytic activity (i.e., a substrate trapping enzyme).

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Assays provided herein include the steps of: a) contacting a sample with a mutant or modified enzyme that binds to the analyte of interest; and b) detecting binding between the analyte or the immediate analyte enzymatic conversion product with the mutant analyte-binding enzyme. The mutant or modified enzyme substantially retains the binding affinity has

5   enhanced binding affinity of the wildtype or unmodified enzyme for the analyte or an immediate analyte enzymatic conversion product but has attenuated catalytic activity.

1.   Analytes

Any analyte that can specifically bind to an enzyme, either as a co-enzyme, a co-factor or a substrate can be assayed by the presently claimed methods. Analytes can be any

10   molecules, including biological macromolecules and small molecules, ligands, anti-ligands and other species. Preferably, the analyte to be assayed is a small molecule. In one embodiment, the small molecule analyte to be assayed is an inorganic molecule. Preferably, the inorganic molecule is an inorganic ion such as a sodium, a potassium, a magnesium, a calcium, a chlorine, an iron, a copper, a zinc, a manganese, a cobalt, an iodine, a molybdenum, a

15   vanadium, a nickel, a chromium, a fluorine, a silicon, a tin, a boron or an arsenic ion.

In another specific embodiment, the small molecule analyte is an organic molecule. Preferably, the organic molecule to be assayed is an amino acid, a peptide containing less than 10 amino acids, a nucleoside, a nucleotide, an oligonucleotide containing less than 10 nucleotides, a vitamin, a monosaccharide, an oligosaccharide containing less than 10

20   monosaccharides or a lipid.

Any amino acids can be assayed by the presently claimed methods. For example, a D- and a L-amino-acid can be assayed. In addition, any building blocks of naturally occurring peptides and proteins including Ala (A), Arg (R), Asn (N), Asp (D), Cys (C), Gln (Q), Glu (E), Gly (G), His (H), Ile (I), Leu (L), Lys (K), Met (M), Phe (F), Pro (P) Ser (S), Thr (T), Trp (W),

25   Tyr (Y) and Val (V) can be assayed. Further, any derivatives of the naturally occurring amino acids, *e.g.*, Hcy as a derivative of Cys, can be assayed.

Any nucleosides can be assayed by the presently claimed methods. Examples of such nucleosides include adenosine, guanosine, cytidine, thymidine and uridine.

Any nucleotides can be assayed by the presently claimed methods. Examples of such

30   nucleotides include AMP, GMP, CMP, UMP, ADP, GDP, CDP, UDP, ATP, GTP, CTP, UTP, dAMP, dGMP, dCMP, dTMP, dADP, dGDP, dCDP, dTDP, dATP, dGTP, dCTP and dTTP. In

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addition, any oligonucleotides containing less than 10 such nucleotides or other nucleotides can be assayed.

Any vitamins can be assayed by the presently claimed methods. For example, water-soluble vitamins such as thiamine, riboflavin, nicotinic acid, pantothenic acid, pyridoxine,

5   biotin, folate, vitamin B$_{12}$ and ascorbic acid can be assayed. Similarly, fat-soluble vitamins such as vitamin A, vitamin D, vitamin E, and vitamin K can be assayed.

Any monosaccharides, whether D- or L-monosaccharides and whether aldoses or ketoses, can be assayed by the presently claimed methods. Examples of monosaccharides include triose such as glyceraldehyde, tetroses such as erythrose and threose, pentoses such as

10   ribose, arabinose, xylose, lyxose and ribulose, hexoses such as allose, altrose, glucose, mannose, gulose, idose, galactose, talose and fructose and heptose such as sedoheptulose.

Any lipids can be assayed by the presently claimed methods. Examples of lipids include triacylglycerols such as tristearin, tripalmitin and triolein, waxes, phosphoglycerides such as phosphatidylethanolamine, phosphatidylcholine, phosphatidylserine,

15   phosphatidylinositol and cardiolipin, sphingolipids such as sphingomyelin, cerebrosides and gangliosides, sterols such as cholesterol and stigmasterol and sterol fatty acid esters. The fatty acids can be saturated fatty acids such as lauric acid, myristic acid, palmitic acid, stearic acid, arachidic acid and lignoceric acid, or can be unsaturated fatty acids such as palmitoleic acid, oleic acid, linoleic acid, linolenic acid and arachidonic acid.

20   In still another specific embodiment, the small molecule to be assayed has a molecular weight that is about or less than 10,000 daltons. More preferably, the small molecule has a molecular weight that is about or less than 5,000 daltons.

Examples of specific analytes that can be assayed by the presently claimed methods include, but are not limited to, Hcy, folate species, cholesterol, glucose, ethanol and uric acid.

25   **2.   Mutant analyte-binding enzymes ("substrate trapping enzymes")**

Any mutant analyte-binding enzyme that substantially retains its binding affinity or has enhanced binding affinity for the analyte or an immediate analyte enzymatic conversion product but has attenuated catalytic activity can be used in the assay. For example, if Hcy is the analyte to be assayed, mutant Hcy-binding enzymes such as mutant cystathionine ß-

30   synthase, mutant methionine synthase, mutant betaine-homocysteine methyltransferase, mutant methioninase and mutant SAH hydrolase can be used.

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Mutant enzymes having the desired specificity can be prepared using routine
mutagenesis methods. Residues to mutate can be identified by systematically mutating
residues to different residues, and identifying those that have the desired reduction in catalytic
activity and retention of binding activity for a particular substrate. Alternatively or

5   additionally, mutations may be based upon predicted or known 3-D structures of enzymes,
including predicted affects of various mutations (see, e.g., Turner, et al. (1998) Nature
Structural Biol. 5:369-376; Ault-Richié, et al. (1994) J. Biol. Chem. 269:31472-31478; Yuan,
et al. (1996) J. Biol. Chem. 271:28009-28016; Williams, et al. (1998) Biochemistry 37:7096;
Steadman, et al. (1998) Biochemistry 37:7089-7095; Finer-Moore, et al. (1998) J. Mol. Biol.

10   276:113-129; Strop, et al. (1997) Protein Sci. 6:2504-2511; Finer-Moore, et al. (1996)
Biochemistry 35:5125-5136; Schiffer, et al. (1995) Biochemistry 34:16279-16287; Costi, et al.
(1996) Biochemistry 35:3944-3949; Graves, et al. (1992) Biochemistry 31:15-21; Carreras, et
al. (1992) Biochemistry 31:6038-6044). Such predictions can be made by those of skill in the
art of computational chemistry. Hence, for any selected enzyme, the mutations need to

15   inactivate catalytic activity but retain binding activity can be determined empirically.

a.   Nucleic acids encoding analyte-binding enzymes

Nucleic acids encoding analyte-binding enzymes can be obtained by methods known in
the art. Known nucleic acid sequences of analyte-binding enzymes can be used in isolating
nucleic acids encoding analyte-binding enzymes from natural or other sources. Alternatively,

20   complete or partial nucleic acids encoding analyte-binding enzymes can be obtained by
chemical synthesis according to the known sequences or obtained from commercial or other
sources.

Eukaryotic cells and prokaryotic cells can serve as a nucleic acid source for the isolation
of nucleic acids encoding analyte-binding enzymes. The DNA can be obtained by standard

25   procedures known in the art from cloned DNA (e.g., a DNA "library"), chemical synthesis,
cDNA cloning, or by the cloning of genomic DNA, or fragments thereof, purified from the
desired cell (see, for example, Sambrook, et al., 1989, Molecular Cloning, A Laboratory
Manual, 2d Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York; Glover,
D.M. (ed.), 1985, DNA Cloning: A Practical Approach, MRL Press, Ltd., Oxford, U.K. Vol. I,

30   II.). Clones derived from genomic DNA can contain regulatory and intron DNA regions in
addition to coding regions; clones derived from cDNA or RNA contain only exon sequences.

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Whatever the source, the gene is generally molecularly cloned into a suitable vector for
propagation of the gene.

In the molecular cloning of the gene from cDNA, cDNA can be generated from totally
cellular RNA or mRNA by methods that are known in the art.. The gene can also be obtained

5   from genomic DNA, where DNA fragments are generated (e.g., using restriction enzymes or by
mechanical shearing), some of which will encode the desired gene. The linear DNA fragments
can then be separated according to size by standard techniques, including but not limited to,
agarose and polyacrylamide gel electrophoresis and column chromatography.

Once the DNA fragments are generated, identification of the specific DNA fragment

10   containing all or a portion of the analyte-binding enzymes gene can be accomplished in a
number of ways.

A preferred method for isolating an analyte-binding enzyme gene is by the polymerase
chain reaction (PCR), which can be used to amplify the desired analyte-binding enzyme
sequence in a genomic or cDNA library or from genomic DNA or cDNA that has not been

15   incorporated into a library. Oligonucleotide primers which hybridize to the analyte-binding
enzyme sequences can be used as primers in PCR.

Additionally, a portion of the analyte-binding enzyme (of any species) gene or its
specific RNA, or a fragment thereof, can be purified (or an oligonucleotide synthesized) and
labeled, the generated DNA fragments may be screened by nucleic acid hybridization to the

20   labeled probe (Benton, W. and Davis, R., 1977, Science 196:180; Grunstein, M. And Hogness,
D., 1975, Proc. Natl. Acad. Sci. U.S.A. 72:3961). Those DNA fragments with substantial
homology to the probe will hybridize. The analyte-binding enzyme nucleic acids can be also
identified and isolated by expression cloning using, for example, anti-analyte-binding enzyme
antibodies for selection.

25   Alternatives to obtaining the analyte-binding enzyme DNA by cloning or amplification
include, but are not limited to, chemically synthesizing the gene sequence itself from the known
analyte-binding enzyme nucleotide sequence or making cDNA to the mRNA which encodes
the analyte-binding enzyme. Any suitable method known to those of skill in the art may be
employed.

30   Once a clone has been obtained, its identity can be confirmed by nucleic acid
sequencing (by methods known in the art) and comparison to known analyte-binding enzyme
sequences. DNA sequence analysis can be performed by techniques known in the art,
including but not limited to, the method of Maxam and Gilbert (1980, Meth. Enzymol. 65:499-

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560), the Sanger dideoxy method (Sanger, F., *et al.*, 1977, *Proc. Natl. Acad. Sci. U.S.A.*
74:5463), the use of T7 DNA polymerase (Tabor and Richardson, U.S. Patent No. 4,795,699),
use of an automated DNA sequenator (*e.g.*, Applied Biosystems, Foster City, CA).

Nucleic acids which are hybridizable to an analyte-binding enzyme nucleic acid, or to a
5   nucleic acid encoding an analyte-binding enzyme derivative can be isolated, by nucleic acid
hybridization under conditions of low, high, or medium stringency (Shilo and Weinberg, 1981,
Proc. Natl. Acad. Sci. USA 78:6789-6792).

b.   Selecting and producing mutant analyte-binding enzymes

Once nucleic acids encoding the analyte-binding enzymes are obtained, these nucleic
10 acids can be mutagenized and screened and/or selected for analyte-binding enzymes that
substantially retain their binding affinity or have enhanced binding affinity for the analyte or an
immediate analyte enzymatic conversion product but have attenuated catalytic activity.
Insertion, deletion, or point mutation(s) can be introduced into nucleic acids encoding the
analyte-binding enzymes. Techniques for mutagenesis known in the art can be used, including,
15 but not limited to, *in vitro* site-directed mutagenesis (Hutchinson, *et al.*, 1978, *J. Biol. Chem*
253:6551), use of TAB® linkers (Pharmacia), mutation-containing PCR primers, *etc.*
Mutagenesis can be followed by phenotypic testing of the altered gene product.

Site-directed mutagenesis protocols can take advantage of vectors that provide single
stranded as well as double stranded DNA, as needed. Generally, the mutagenesis protocol with
20 such vectors is as follows. A mutagenic primer, *i.e.*, a primer complementary to the sequence
to be changed, but including one or a small number of altered, added, or deleted bases, is
synthesized. The primer is extended *in vitro* by a DNA polymerase and, after some additional
manipulations, the now double-stranded DNA is transfected into bacterial cells. Next, by a
variety of methods, the desired mutated DNA is identified, and the desired protein is purified
25 from clones containing the mutated sequence. For longer sequences, additional cloning steps
are often required because long inserts (longer than 2 kilobases) are unstable in those vectors.
Protocols are known to one skilled in the art and kits for site-directed mutagenesis are widely
available from biotechnology supply companies, for example from Amersham Life Science,
Inc. (Arlington Heights, IL) and Stratagene Cloning Systems (La Jolla, CA).

30   Information regarding the structural-functional relationship of the analyte-binding
enzymes can be used in the mutagenesis and selection of analyte-binding enzymes that
substantially retain their binding affinity or have enhanced binding affinity for the analyte or an

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immediate analyte enzymatic conversion product but have attenuated catalytic activity. For

example, mutants can be made in the enzyme's binding site for its co-enzyme, co-factor, a non-

analyte substrate, or in the mutant enzyme's catalytic site, or a combination thereof.

Once a mutant analyte-binding enzyme with desired properties, i.e., substantially

5   retaining its binding affinity or having enhanced binding affinity for the analyte or an

immediate analyte enzymatic conversion product but has attenuated catalytic activity, is

identified, such mutant analyte-binding enzyme can be produced by any methods known in the

art including recombinant expression, chemical synthesis or a combination thereof. Preferably,

the mutant analyte-binding enzyme is obtained by recombinant expression.

10   For recombinant expression, the mutant analyte-binding enzyme gene or portion thereof

is inserted into an appropriate cloning vector for expression in a particular host cell. A large

number of vector-host systems known in the art may be used. Possible vectors include, but are

not limited to, plasmids or modified viruses, but the vector system must be compatible with the

host cells used. Such vectors include, but are not limited to, bacteriophages such as lambda

15   derivatives, or plasmids such as pBR322 or pUC plasmid derivatives or the Bluescript vector

(Stratagene). The insertion into a cloning vector can, for example, be accomplished by ligating

the DNA fragment into a cloning vector which has complementary cohesive termini. If,

however, the complementary restriction sites used to fragment the DNA are not present in the

cloning vector, the ends of the DNA molecules can be enzymatically modified. Alternatively, a

20   desired site can be produced by ligating sequences of nucleotides (linkers) onto the DNA

termini; these ligated linkers can include specific oligonucleotides encoding restriction

endonuclease recognition sequences. Recombinant molecules can be introduced into host cells

via transformation, transfection, infection, electroporation, etc., so that many copies of the gene

sequence are generated.

25   In an alternative method, the desired gene can be identified and isolated after insertion

into a suitable cloning vector in a "shot gun" approach. Enrichment for the desired gene, for

example, by size fractionation, can be done before insertion into the cloning vector.

In specific embodiments, transformation of host cells with recombinant DNA molecules

that incorporate the isolated mutant analyte-binding enzyme gene, cDNA, or synthesized DNA

30   sequence enables generation of multiple copies of the gene. Thus, the gene can be obtained in

large quantities by growing transformants, isolating the recombinant DNA molecules from the

transformants and, when necessary, retrieving the inserted gene from the isolated recombinant

DNA.

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The nucleotide sequence coding for a mutant analyte-binding enzyme or a functionally active analog or fragment or other derivative thereof, can be inserted into an appropriate expression vector, e.g., a vector which contains the necessary elements for the transcription and translation of the inserted protein-coding sequence. The necessary transcriptional and

5   translational signals can also be supplied by the native mutant analyte-binding enzyme gene and/or its flanking regions. A variety of host-vector systems can be utilized to express the protein-coding sequence. These systems include but are not limited to mammalian cell systems infected with virus (e.g., vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (e.g., baculovirus); microorganisms such as yeast containing yeast vectors, or bacteria

10 transformed with bacteriophage, DNA, plasmid DNA, or cosmid DNA. The expression elements of vectors vary in their strengths and specificities. Depending on the host-vector system utilized, suitable transcription and translation elements can be used.

The methods previously described for the insertion of DNA fragments into a vector can be used to construct expression vectors containing a chimeric gene containing appropriate

15 transcriptional/translational control signals and the protein coding sequences. These methods can include in vitro recombinant DNA and synthetic techniques and in vivo recombinants (genetic recombination). Expression of a nucleic acid sequence encoding a mutant analyte-binding enzyme or peptide fragment can be regulated by a second nucleic acid sequence so that the mutant analyte-binding enzyme or peptide is expressed in a host transformed with the

20 recombinant DNA molecule. For example, expression of a mutant analyte-binding enzyme can be controlled by a promoter/enhancer element as is known in the art. Promoters which can be used to control a mutant analyte-binding enzyme expression include, but are not limited to, the SV40 early promoter region (Bernoist and Chambon, 1981, Nature 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, et al., 1980, Cell

25 22:787-797), the herpes thymidine kinase promoter (Wagner, et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster, et al., 1982, Nature 296:39-42); prokaryotic expression vectors such as the β-lactamase promoter (Villa-Kamaroff, et al., 1978, Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731), or the tac promoter (DeBoer, et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:21-25); see also "Useful proteins from

30 recombinant bacteria" in Scientific American, 1980, 242:74-94; promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADC (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter, and certain animal transcriptional control regions.

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For example, a vector can be used that contains a promoter operably linked to a nucleic acid encoding a mutant analyte-binding enzyme, one or more origins of replication, and, optionally, one or more selectable markers (*e.g.*, an antibiotic resistance gene).

In a specific embodiment, an expression construct is made by subcloning a mutant

5   analyte-binding enzyme coding sequence into the *Eco*RI restriction site of each of the three pGEX vectors (Glutathione S-Transferase expression vectors; see, *e.g.*, Smith and Johnson, 1988, *Gene* 7:31-40). This allows for the expression of a mutant analyte-binding enzyme product from the subclone in the correct reading frame.

Expression vectors containing a mutant analyte-binding enzyme gene inserts can be

10   identified by three general approaches: (a) nucleic acid hybridization, (b) presence or absence of "marker" gene functions, and (c) expression of inserted sequences. In the first approach, the presence of a mutant analyte-binding enzyme gene inserted in an expression vector can be detected by nucleic acid hybridization using probes containing sequences that are homologous to an inserted mutant analyte-binding enzyme gene. In the second approach, the recombinant

15   vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (*e.g.*, thymidine kinase activity, resistance to antibiotics, transformation phenotype, occlusion body formation in baculovirus, etc.) caused by the insertion of a mutant analyte-binding enzyme gene in the vector. For example, if the mutant analyte-binding enzyme gene is inserted within the marker gene sequence of the vector,

20   recombinants containing the mutant analyte-Binding enzyme insert can be identified by the absence of the marker gene function. In the third approach, recombinant expression vectors can be identified by assaying the mutant analyte-binding enzyme product expressed by the recombinant. Such assays can be based, for example, on the physical or functional properties of the mutant analyte-binding enzyme in *in vitro* assay systems, *e.g.*, binding with anti-mutant

25   analyte-binding enzyme antibody.

Once a particular recombinant DNA molecule is identified and isolated, several methods known in the art can be used to propagate it. Once a suitable host system and growth conditions are established, recombinant expression vectors can be propagated and prepared in quantity. As previously explained, the expression vectors which can be used include, but are

30   not limited to, the following vectors or their derivatives: human or animal viruses such as vaccinia virus or adenovirus; insect viruses such as baculovirus; yeast vectors; bacteriophage vectors (*e.g.*, lambda), and plasmid and cosmid DNA vectors, to name but a few.

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In addition, a host cell strain can be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Expression from certain promoters can be elevated in the presence of certain inducers; thus, expression of the genetically engineered mutant analyte-binding enzyme can be controlled.

5   Furthermore, different host cells have characteristic and specific mechanisms for the translational and post-translational processing and modification (*e.g.*, glycosylation, phosphorylation) of proteins. Appropriate cell lines or host systems can be chosen to ensure the desired modification and processing of the foreign protein expressed. For example, expression in a bacterial system can be used to produce an unglycosylated core protein product.

10   Expression in yeast will produce a glycosylated product. Expression in appropriate animal cells can be used to ensure "native" glycosylation of a heterologous protein. Furthermore, different vector/host expression systems can effect processing reactions to different extent.

3.   Sample collection

Any sample can be assayed for an analyte using the above-described methods. In one

15   embodiment, the sample being assayed is a biological sample from a mammal, particularly a human, such as a biological fluid or a biological tissue. Biological fluids, include, but are not limited to, are urine, blood, plasma, serum, saliva, semen, stool, sputum, hair and other keratinous samples, cerebral spinal fluid, tears, mucus and amniotic fluid. Biological tissues contemplated include, but are not limited to, aggregates of cells, usually of a particular kind

20   together with their intercellular substance that form one of the structural materials of a human, animal, plant, bacterial, fungal or viral structure, including connective, epithelium, muscle and nerve tissues, organs, tumors, lymph nodes, arteries and individual cell(s). In one specific embodiment, the body fluid to be assayed is urine. In another specific embodiment, the body fluid to be assayed is blood. Preferably, the blood sample is further separated into a plasma or

25   sera fraction.

Serum or plasma can be recovered from the collected blood by any methods known in the art. In one specific embodiment, the serum or plasma is recovered from the collected blood by centrifugation. Preferably, the centrifugation is conducted in the presence of a sealant having a specific gravity greater than that of the serum or plasma and less than that of the blood

30   corpuscles which will form the lower, whereby upon centrifugation, the sealant forms a separator between the upper serum or plasma layer and the lower blood corpuscle layer. The sealants that can be used in the processes include, but are not limited to, styrene resin powders

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(Japanese Patent Publication No. 38841/1973), pellets or plates of a hydrogel of a crosslinked polymer of 2-hydroxyethyl methacrylate or acrylamide (U.S. Patent No. 3,647,070), beads of polystyrene bearing an antithrombus agent or a wetting agent on the surfaces (U.S. Patent No. 3,464,890) and a silicone fluid (U.S. Patent Nos. 3,852,194 and 3,780,935). In a preferred

5   embodiment, the sealant is a polymer of unsubstituted alkyl acrylates and/or unsubstituted alkyl methacrylates, the alkyl moiety having not more than 18 carbon atoms, the polymer material having a specific gravity of about 1.03 to 1.08 and a viscosity of about 5,000 to 1,000,000 cps at a shearing speed of about 1 second^{-1} when measured at about 25°C (U.S. Patent No. 4,140,631).

10   In another specific embodiment, the serum or plasma is recovered from the collected blood by filtration. Preferably, the blood is filtered through a layer of glass fibers with an average diameter of about 0.2 to 5 μ and a density of about 0.1 to 0.5 g./cm^3, the total volume of the plasma or serum to be separated being at most about 50% of the absorption volume of the glass fiber layer; and collecting the run-through from the glass fiber layer which is plasma

15   or serum (U.S. Patent No. 4,477,575). Also preferably, the blood is filtered through a layer of glass fibers having an average diameter 0.5 to 2.5 μ impregnated with a polyacrylic ester derivative and polyethylene glycol (U.S. Patent No. 5,364,533). More preferably, the polyacrylic ester derivative is poly(butyl acrylate), poly(methyl acrylate) or poly(ethyl acrylate), and (a) poly(butyl acrylate), (b) poly(methyl acrylate) or poly(ethyl acrylate) and

20   (c) polyethylene glycol are used in admixture at a ratio of (10-12):(1-4):(1-4).

In still another specific embodiment, the serum or plasma is recovered from the collected blood by treating the blood with a coagulant containing a lignan skeleton having oxygen-containing side chains or rings (U.S. Patent No. 4,803,153). Preferably, the coagulant contains a lignan skeleton having oxygen-containing side chains or rings, e.g., d-sesamin, l-

25   sesamin, paulownin, d-asarinin, l-asarinin, 2α-paulownin, 6α-paulownin, pinoresinol, d-eudesmin, l-pinoresinol ß-D-glucoside, l-pinoresinol, l-pinoresinol monomethyl ether ß-D-glucoside, epimagnolin, lirioresinol-B, syringaresinol (dl), lirioresinonB-dimethyl ether, phillyrin, magnolin, lirioresinol-A, 2α, 6α-d-sesamin, d-diaeudesmin, lirioresinol-C dimethyl ether (ddiayangambin) and sesamolin. More preferably, the coagulant is used in an amount

30   ranging from about 0.01 to 50 g per 1 l of the blood.

C.   METHODS FOR ASSAYING HOMOCYSTEINE

Also provided herein is a method for assaying Hcy in a sample. The method includes at least the steps of: a) contacting the sample with a mutant Hcy-binding enzyme, the mutant enzyme substantially retains its binding affinity or has enhanced binding affinity for Hcy or an

5   immediate Hcy enzymatic conversion product but has attenuated catalytic activity; and b) detecting binding between the Hcy or the immediate Hcy enzymatic conversion product with the mutant Hcy-binding enzyme.

1.   Homocysteine metabolism

Homocysteine is an intermediary amino acid produced when methionine is metabolized

10   to cysteine. There are two routes by which homocysteine produced in the body is rapidly metabolized: (1) condensation with serine to form cystathione or (2) conversion to methionine.

As discussed above, homocysteine levels in biological samples are of clinical significance. Homocysteine plays a role sulfhydryl amino acid metabolism; its accumulation may be indicative of various disorders occurring in these pathways, including in particular

15   inborn errors of metabolism. Thus, for example homocystinuria (an abnormal build-up of homocysteine in the urine) is a disorder of amino acid metabolism resulting from deficiencies in the enzymes cystathione β-synthetase or methyltetrahydrofolic acid methyltransferase, which catalyses the methylation of homocysteine to methionine.

- In the second pathway, which is illustrated as follows:

20
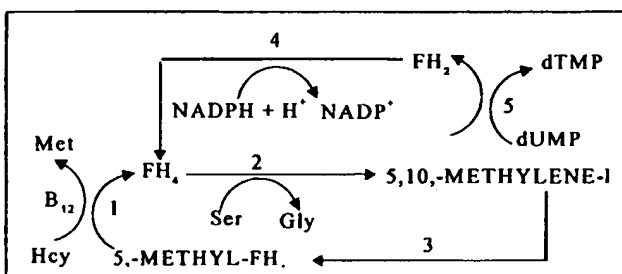
where: 1 is methylene synthase; 2 is tetrahydrofolate (FH_4) methyltransferase; 3 is

30   methylenetetrahydrofolate reductase; 4 is dihydrofolate reductase; 5 is thymidylate synthase; FH_4 is tetrahydrofolate and FH_2 is dihydrofolate, homocysteine levels are related, among other things, to folate levels and also vitamin B_{12} levels. The various enzymes in these pathways may be assessed and correlated with homocysteine levels.

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Sulfhydryl amino acid metabolism is closely linked to that of folic acid and vitamin B_{12} (cobalamin), which act as substrates or co-factors in the various transformations involved. Homocysteine accumulation can be an indicator of malfunction of cobalamin or folate dependent enzymes, or other disorders or diseases related to cobalamin or folate metabolism.

5   Homocysteine metabolism also may be affected by anti-folate drugs, such as methotrexate, administered to treat disorders, such as cancer and asthma, since homocysteine conversion to methionine relies on a reaction requiring S-methyl tetrahydrofolate as the methyl donor. Monitoring of homocysteine has therefore also been proposed in the management of malignant disease treatment with anti-folate drugs. More recently, elevated levels of

10   homocysteine in the blood have been correlated with the development of atherosclerosis (see Clarke, et al., New Eng. J. Med. 324:1149-1155 (1991)) and even moderate homocysteinemia is a risk factor for cardiac and vascular diseases. Measurement of plasma or blood levels of homocysteine is thus also of importance in the diagnosis and treatment of vascular disease.

2.   Mutant Hcy-binding enzymes

15   Any mutant Hcy-binding enzymes that substantially retain their binding affinity or have enhanced binding affinity for Hcy or an immediate Hcy enzymatic conversion product but have attenuated catalytic activity can be used in the Hcy assay. Examples of such mutant Hcy-binding enzyme include mutant cystathionine ß-synthase, mutant methionine synthase, mutant betaine-homocysteine methyltransferase, mutant methioninase and mutant SAH hydrolase.

20   a.   Nucleic acids encoding Hcy-binding enzymes

Nucleic acids encoding Hcy-binding enzymes can be obtained by methods known in the art. Additional nucleic acid molecules encoding such enzymes are known and the molecules or sequences thereof are publicly available. If the molecules are available they can be used; alternatively the known sequences can be used to obtain clones from selected or desired

25   sources. For example, the nucleic acid sequences of Hcy-binding enzymes, such as cystathionine ß-synthase, methionine synthase, betaine-homocysteine methyltransferase, methioninase and SAH hydrolase, can be used in isolating nucleic acids encoding Hcy-binding enzymes from natural sources. Alternatively, nucleic acids encoding Hcy-binding enzymes can be obtained by chemical synthesis according to the known sequences.

30   In one embodiment, the nucleic acid molecules containing sequences of nucleotides with the following GenBank accession Nos. can be used in obtaining nucleic acid encoding

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SAH hydrolase: AF129871 (Gossypium hirsutum); AQ003753 (Cryptosporidium parvum);

AF105295 (Alexandrium fundyense); AA955402 (Rattus norvegicus); AA900229 (Rattus

norvegicus); AA874914 (Rattus norvegicus); AA695679 (Drosophila melanogaster ovary);

AA803942 (Drosophila melanogaster ovary; AI187655 (Manduca sexta male antennae);

5   U40872 (Trichomonas vaginalis); AJ007835 (Xenopus Laevis); AF080546 (Anopheles

gambiae); AI069796 (T. cruzi epimastigote); Z97059 (Arabidopsis thaliana); AF059581

(Arabidopsis thaliana); U82761 (Homo sapiens); AA754430 (Oryza sativa); D49804

(Nicotiana tabacum); D452u4 (Nicotiana tabacum); X95636 (D. melanogaster); T18277

(endosperm Zea mays); R75259 (Mouse brain); Z26881 (C. roseus); X12523 (D. discoideum);

10   X64391 (Streptomyces fradiae); W21772 (Maize Leaf); AH003443 (Rattus norvegicus);

U14963 (Rattus norvegicus); U14962 (Rattus norvegicus); U14961 (Rattus norvegicus);

U14960 (Rattus norvegicus); U14959 (Rattus norvegicus); U14937 (Rattus norvegicus);

U14988 (Rattus norvegicus); U14987 (Rattus norvegicus); U14986 (Rattus norvegicus);

U14985 (Rattus norvegicus); U14984 (Rattus norvegicus); U14983 (Rattus norvegicus);

15   U14982 (Rattus norvegicus); U14981 (Rattus norvegicus); U14980 (Rattus norvegicus);

U14979 (Rattus norvegicus); U14978 (Rattus norvegicus); U14977 (Rattus norvegicus);

U14976 (Rattus norvegicus); U14975 (Rattus norvegicus); L32836 (Mus musculus); L35559

(Xenopus laevis); Z19779 (Human foetal Adrenals tissue); L23836 (Rhodobacter capsulatus);

M15185 (Rat); L11872 (Triticum aestivum); M19937 (Slime mold (D. discoideum); M80630

20   (Rhodobacter capsulatus). Preferably, the nucleic acid molecules containing nucleotide

sequences with the GenBank accession Nos. M61831-61832 can be used in obtaining nucleic

acid encoding SAH hydrolase (SEQ ID No. 1; see also Coulter-Karis and Hershfield, *Ann.*

Hum. Genet., 53(2):169-175 (1989)). Also preferably, the nucleic acid molecule containing the

sequence of nucleotides or encoding the amino acids set forth in SEQ ID No. 3 can be used (see

25   also U.S. Patent No. 5,854,023).

In another specific embodiment, the nucleic acid molecules containing sequences of

nucleotides with the following GenBank accession Nos. can be used in obtaining nucleic acid

encoding methionine synthase: AI547373 (Mesembryanthemum crystallinum); AI507856

(COBALAMINE-INDEPENDENT METHIONINE SYNTHASE); AI496185

30   (COBALAMINE-INDEPENDENT METHIONINE SYNTHASE); AI496016

(COBALAMINE-INDEPENDENT METHIONINE SYNTHASE); AI495904

(COBALAMINE-INDEPENDENT METHIONINE SYNTHASE); AI495702; AI495399;

AI461276 (COBALAMINE-INDEPENDENT METHIONINE SYNTHASE); AI460827

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(COBALAMINE-INDEPENDENT METHIONINE SYNTHASE); AI460549; AI443293; AI443243 (COBALAMINE-INDEPENDENT METHIONINE SYNTHASE); AI443242 (COBALAMINE-INDEPENDENT METHIONINE SYNTHASE); AI442736 (COBALAMINE-INDEPENDENT METHIONINE SYNTHASE); AI442546; AI442173

5   (COBALAMINE-INDEPENDENT METHIONINE SYNTHASE); AI442136 (COBALAMINE-INDEPENDENT METHIONINE SYNTHASE); AI441314; AI440982; AI438053; AI416939 (COBALAMINE-INDEPENDENT METHIONINE SYNTHASE); AI416601; AI391967 (Conidial Neurospora crassa); AF034214 (Rattus norvegicus); U77388 (Chlamydomonas moewusii); AF093539 (Zea mays); U97200 (Arabidopsis thaliana); U36197

10   (Chlamydomonas reinhardtii); AF025794 (Homo sapiens); AJ222785 (Hordeum vulgare); Z49150 (C. blumei kinetoplast met gene); AB004651 (Hyphomicrobium methylovorum gene); AA661438 (Maize Leaf); AA661023 (Medicago truncatula); AA660965 (Medicago truncatula); AA660880 (Medicago truncatula); AA660780 (Medicago truncatula); AA660708 (Medicago truncatula); AA660643 (Medicago truncatula); AA660558 (Medicago truncatula);

15   AA660475 (Medicago truncatula); AA660444 (Medicago truncatula); AA660382 (Medicago truncatula); AA660310 (Medicago truncatula); AA660241 (Medicago truncatula); U75743 (Human); AA389835 (Arabidopsis thaliana); U84889 (Mesembryanthemum crystallinum); U73338 (Human); AA054818 (Maize Leaf); AA030695 (Maize Leaf); X83499 (C. roseus); U15099 (Saccharomyces cerevisiae (MET6)); J02804 (*E. coli* speED operon speE and speD

20   genes); M87625 (Escherichia coli); J04975 (*E. coli*). Preferably, the nucleic acid molecules containing sequences of nucleotides with GenBank accession Nos. U75743 (SEQ ID No. 4) and U73338 (SEQ ID No. 6) can be used to obtain nucleic acid encoding methionine synthase.

In still another specific embodiment, nucleic acid molecules containing sequences of nucleotides with the following GenBank accession Nos. can be used in obtaining nucleic acid

25   encoding cystathionine ß-synthase: AI584826 (Zebrafish L19501); AI566920 (Homo sapiens); AI558544 (Zebrafish); AI529762 (Sugano mouse liver); AI528420 (Sugano mouse liver); AI494445 (Homo sapiens); AI500425 (Homo sapiens); AI421007 (Homo sapiens); AI369768 (Homo sapiens); AI368618 (Homo sapiens); AI312384 (Homo sapiens); AI266220 (Homo sapiens); AI307196 (Homo sapiens); R85449 (Homo sapiens); R84640 (Homo sapiens);

30   AI371928 (Homo sapiens); AI281692 (Homo sapiens); AI198353 (Homo sapiens); AI222601 (Homo sapiens); AI188666 (Soares placenta); AI088293 (Soares Homo sapiens); AI039450 (Homo sapiens); AA995138 (Homo sapiens); AI053744 (Homo sapiens); AA921824 (Homo sapiens); AA876324 (Homo sapiens); AA218777 (neuronal precursor Homo sapiens);

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AA243110 (neuronal precursor Homo sapiens); AA232188 (neuronal precursor Homo sapiens); AA227066 (neuronal precursor Homo sapiens); AA180443 (HeLa cell Homo sapiens); AA179769 (HeLa cell Homo sapiens); AA620410 (lung carcinoma Homo sapiens); AA173243 (neuroepithelium Homo sapiens); AA173133 (neuroepithelium Homo sapiens);

5   AA811740 (Homo sapiens); AA659341 (Homo sapiens); AA729802 (Homo sapiens); AA063294 (corneal stroma); AA063180 (corneal stroma); AA701200 (fetal liver spleen); AA699637 (fetal liver spleen); AA652920 (Homo sapiens); AA430416 (ovary tumor); AA430367 (ovary tumor); AA642534 (Homo sapiens); AA618538 (Homo sapiens); AA548257 (Homo sapiens); AA554953 (Homo sapiens); AA548561 (Homo sapiens);

10   AA136426 (lung carcinoma); AA136339 (lung carcinoma); AA057714 (corneal stroma); AA260332 (mouse NML Mus musculus); AA239916 (mouse NML Mus musculus); AA239480 (mouse NML Mus musculus); AA096780 (mouse lung); AA105071 (mouse kidney); N76209 (fetal liver spleen); N54505 (fetal liver spleen); AA171542 (neuroepithelium); AA171511 (neuroepithelium); S78267 (human, homocystinuria patient 12,

15   skin fibroblasts); AA057541 (corneal stroma); N50670 (multiple sclerosis); N29067 (melanocyte); T28038 (Human Brain Homo sapiens); H11280 (infant brain); R78956 (placenta); R38394 (infant brain); R35233 (placenta); T91706 (lung); T70457 (liver); T69322 (liver); T69248 (liver); L00972 (Human). Preferably, a nucleic acid molecule containing sequences of nucleotides set forth in SEQ ID No. 8 can be used in obtaining nucleic acid

20   encoding cystathionine ß-synthase (see also U.S. Patent No. 5,523,225).

In yet another specific embodiment, the nucleotide sequences with the following GenBank accession Nos. can be used in obtaining nucleic acid encoding betaine homocysteine S-methyltransferase: AI629131 (Zebrafish); AI601766 (Zebrafish); NM001713 (Homo sapiens); AH007531 (Homo sapiens); AF118378 (Homo sapiens); AF118377 (Homo sapiens);

25   AF118376 (Homo sapiens); AF118375 (Homo sapiens); AF118374 (Homo sapiens); AF118373 (Homo sapiens); AF118372 (Homo sapiens); AF118371 (Homo sapiens); AI550844 (mouse lung); AI529920 (mouse liver); AI529834 (mouse liver); AI529135 (mouse liver); AI527147 (mouse liver); AI527097 (mouse liver); AI497458 (Zebrafish); AI497232 (Zebrafish); AI496988 (Zebrafish); AI496904 (Zebrafish); AI496821 (Zebrafish); AI496747

30   (Zebrafish); AI471640 (Homo sapiens); AA901407 (Rattus norvegicus); AI390284 (mouse); AI244216 (Homo sapiens); AI316045 (mouse liver); AI303938 (mouse liver); AI303911 (mouse liver); AI303222 (mouse liver); AI287146 (mouse liver); AI287008 (mouse liver); AI286878 (mouse liver); AI266927 (mouse liver); AI256283 (mouse liver); AI227233 (mouse

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liver); AI227053 (mouse liver); U50929 (Human); U53421 (Sus scrofa); AI132261 (mouse
liver); AI132254 (mouse liver); AI118276 (mouse liver); AI116416 (mouse liver); AI115840
(mouse kidney); AI115838 (mouse kidney); AI048111 (mouse liver); AI043140 (mouse liver);
AA989805 (mouse kidney); AA986591 (mouse kidney); AA986590 (mouse kidney);

5   AA985983 (mouse liver); AA755243 (mouse diaphragm); AF038870 (Rattus norvegicus);
AA693837 (fetal liver); U96133 ((Rattus norvegicus). Preferably, the nucleotide sequences
with the GenBank accession No. AH007531 can be used in obtaining nucleic acid encoding
betaine homocysteine S-methyltransferase (SEQ ID No. 10; see also Garrow, *J. Biol. Chem.*,
271(37):22831-8 (1996)).

10   In yet another specific embodiment, the nucleotide sequences described in U.S. Patent
No. 5,891,704 (SEQ ID No. 11) and the nucleotide sequences with the GenBank Accession
No. L43133 (SEQ ID No. 13) (Hori, *et al.*, *Cancer Res.*, 56(9):2116-22 (1996)) can be used in
obtaining nucleic acid encoding methioninase.

b.   Selecting and producing Hcy-binding enzymes

15   Once nucleic acids encoding Hcy-binding enzymes are obtained, these nucleic acids can
be mutagenized and screened and/or selected for Hcy-binding enzymes that substantially retain
their binding affinity or have enhanced binding affinity for Hcy or an immediate Hcy
enzymatic conversion product but have attenuated catalytic activity. Insertion, deletion, or
point mutation(s) can be introduced into nucleic acids encoding Hcy-binding enzymes

20   according to methods known to those of skill in the art, and, particularly, those described in
Section C2. herein.

           Information regarding the structural-functional relationship of the Hcy-binding enzymes
can be used in the mutagenesis and selection of Hcy-binding enzymes that substantially retain
their binding affinity or have enhanced binding affinity for Hcy or an immediate Hcy

25   enzymatic conversion product but have attenuated catalytic activity. For example, mutants can
be made in the enzyme's binding site for its co-enzyme, co-factor, a non-Hcy substrate, or in
the mutant enzyme's catalytic site, or a combination thereof.

           In one specific embodiment, wherein cystathionine ß-synthase is mutagenized, mutants
can be made in cystathionine ß-synthase's binding site for pyridoxal 5'-phosphate or L-serine,

30   or a combination thereof (Kim, *et al.*, *Proc. Nat. Acad. Sci.*, 71(2):4821-4825 (1974)). For
example, Lys119 of human cystathionine ß-synthase can be deleted or mutated, preferably to a
non-charged or acidic amino acid residue (Kery, *et al.*, *Biochemistry*, 38(9):2716-24 (1999)).

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In another specific embodiment, wherein methionine synthase is mutagenized, mutants can be made in methionine synthase's binding site for vitamin B_{12} or 5-methyltetrahydrofolate (5-CH_3-THF), or a combination thereof. For example, Asp946, Glu1097, Arg1134, Ala1136, Gly1138, Tyr1139 and Tyr1189 of human methionine synthase can be deleted or mutated,

5   preferably to a different type of amino acid residue, *i.e.*, Asp and Glu are changed to non-charged or basic residue, Arg is changed to non-charged or acidic residue, Ala and Gly are changed to charged residue or non-charged residue with larger sidechain, and Tyr is charged to residue without an aromatic sidechain (Dixon, *et al.*, *Structure*, 4(11):1263-75 (1996)). Preferably, *E. coli.* methionine synthase with amino acid sequence set forth in SEQ ID No. 3,

10   containing His759Gly, Asp757Glu, Asp757Asn, or Ser810Ala is used in the Hcy assay (Amaratunga, *et al.*, *Biochemistry*, 35(7):2453-63 (1996))

In still another embodiment, wherein SAH hydrolase is mutagenized, mutants can be made in SAH hydrolase's binding site for NAD^+, or mutation(s) in the mutant SAH hydrolase's catalytic site, *e.g.*, the 5'-hydrolytic catalytic site, or a combination thereof.

15   In yet another embodiment, wherein betaine-homocysteine methyltransferase is mutagenized, mutants can be made in betaine-homocysteine methyltransferase's binding site for Zn^+ or betaine. For example, Cys299 and Cys300 of human betaine-homocysteine methyltransferase can be deleted or mutated, preferably to amino acid residue without -SH sidechain, *e.g.*, Serine (Millian and Garrow, *Arch. Biochem. Biophys.*, 356(1):93-8 (1998)).

20   In yet another specific embodiment, wherein methioninase is mutagenized, mutants can be made in methioninase's binding site for R'SH which represents an alkanethiol or a substituted thiol (Ito, *et al.*, *J. Biochem.*, (Tokyo) 80(6):1327-34 (1976)).

Once a mutant Hcy-binding enzyme with desired properties, *i.e.*, substantially retaining its binding affinity or having enhanced binding affinity for Hcy or an immediate Hcy enzymatic

25   conversion product but has attenuated catalytic activity, is identified, such mutant Hcy-binding enzyme can be produced by any methods known in the art including recombinant expression, chemical synthesis or a combination thereof as described in Section B. Preferably, the mutant Hcy-binding enzyme is obtained by recombinant expression.

c.   **Mutant SAH hydrolase and nucleic acids encoding the mutant SAH**

30   **hydrolase**

SAH hydrolase from mammalian sources are homotetramer of approximate molecular weight of 180-190 KD. The enzyme contains 4 molecules of tightly-bound NAD^+ as a co-

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enzyme. The catalytic mechanism of the enzyme in the hydrolytic direction includes two consecutive reactions, *i.e.*, the 3'-oxidation of the substrate to 3'-keto in concomitant with the reduction of the enzyme-bound NAD$^+$ to NADH, and followed by the 5'-hydrolysis to release the reaction products Hcy and Ado (Refsum, *et al.*, *Clin. Chem.*, 31:624-628 (1985)). The C-

5   terminal regions of all known SAH hydrolase are extremely conserved and contain essential amino acid residues to the enzyme catalysis. The crystal structure of human SAH hydrolase in complex with a substrate analog inhibitor was recently determined. This x-ray structure of SAH hydrolase indicates that at least twenty amino acid residues are directly or indirectly interacting with the substrate analog inhibitor and co-enzyme NAD$^+$. Mutations of those amino

10   acid residues that are involved directly or indirectly in the substrate binding and catalysis can readily be made by site-directed mutagenesis, and the sequence of the resulting mutant enzyme can be confirmed by comparing the mutant SAH hydrolase DNA sequence with the sequence of the wild type enzyme to ensure no other mutations are introduced to the specific mutant enzyme.

15   Provided herein is a substantially purified mutant SAH hydrolase that substantially retains its binding affinity or has enhanced binding affinity for homocysteine (Hcy) or SAH but has attenuated catalytic activity.

     In one specific embodiment, the attenuated catalytic activity of the mutant SAH hydrolase is caused by mutation(s) in the mutant SAH hydrolase's binding site for NAD$^+$, or

20   mutation(s) in the mutant SAH hydrolase's catalytic site or a combination thereof.

     In another specific embodiment, the mutant SAH hydrolase has attenuated 5'-hydrolytic activity but substantially retains its 3'-oxidative activity.

     In still another specific embodiment, the mutant SAH hydrolase irreversibly binds SAH.

     In yet another specific embodiment, the mutant SAH hydrolase has a Km for SAH that

25   is about or less than 10.0 µM. Preferably, the mutant SAH hydrolase has a Km for SAH that is about 1.0 µM or less than 1.0 µM.

     In yet another specific embodiment, the mutant SAH hydrolase has a Kcat for SAH that is about or less than 0.1 S^{-1}.

     In yet another specific embodiment, the mutant SAH hydrolase has one or more

30   insertion, deletion, or point mutation(s). Preferably, the mutant SAH hydrolase is derived from the sequence of amino acids set forth in SEQ ID No. 1 or encoded by the sequence of nucleotides set forth in SEQ ID No. 2 but has one or more of the following mutations: Phe 302 to Ser (F302S), Lys 186 to Ala (K186A), His 301 to Asp (H301D), His 353 to Ser (H353S),

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Arg 343 to Ala (R343A), Asp 190 to Ala (D190A), Phe 82 to Ala (F82A), Thr 157 to Leu

(T157L), Cys 195 to Asp (C195D), Asn 181 to Asp (N181D), and deletion of Tyr 432 (Δ432).

Also more preferably, the mutant SAH hydrolase is a derived sequence of amino acids set forth

in SEQ ID No. 1 or encoded by the sequence of nucleotides set forth in SEQ ID No. 2 and has a

5   combination of Arg 431 to Ala (R431A) and Lys 426 to Arg (K426R) mutations. The nucleic

acid molecules contemplated also include those that have conservative amino acid changes, and

include those that hybridize along their full length to the coding portion of the sequence of

nucleotides set forth in SEQ ID No. 2, under medium stringency, or preferably high stringency,

such that the encoded protein retains ability to bind to the selected analyte without substantial

10   conversion of the analyte.

       Also provided herein is an isolated nucleic acid fragment, either DNA or RNA, that

includes a sequence of nucleotides encoding a mutant S-adenosylhomocysteine (SAH)

hydrolase, the mutant SAH hydrolase substantially retains its binding affinity or has enhanced

binding affinity for homocysteine (Hcy) or SAH but has attenuated catalytic activity.

15   In one specific embodiment, the isolated nucleic acid fragment encodes a mutant SAH

hydrolase wherein the attenuated catalytic activity is caused by mutation(s) in the mutant SAH

hydrolase's binding site for NAD^+, or mutation(s) in the mutant SAH hydrolase's catalytic site

or a combination thereof.

       In another specific embodiment, the isolated nucleic acid fragment encodes a mutant

20   SAH hydrolase wherein the mutant SAH hydrolase has attenuated 5'-hydrolytic activity but

substantially retains its 3'-oxidative activity.

       In still another specific embodiment, the isolated nucleic acid fragment encodes a

mutant SAH hydrolase wherein the mutant SAH hydrolase irreversibly binds SAH.

       In yet another specific embodiment, the isolated nucleic acid fragment encodes a mutant

25   SAH hydrolase wherein the mutant SAH hydrolase has a Km for SAH that is about or less than

10.0 μM. Preferably, the isolated nucleic acid fragment encodes a mutant SAH hydrolase

wherein the mutant SAH hydrolase has a Km for SAH that is about 1.0 μM or less than 1.0 μM.

       In yet another specific embodiment, the isolated nucleic acid fragment encodes a mutant

SAH hydrolase wherein the mutant SAH hydrolase has a Kcat for SAH that is about or less

30   than 0.1 S^{-1}.

       In yet another specific embodiment, the isolated nucleic acid fragment encodes a mutant

SAH hydrolase wherein the mutant SAH hydrolase has one or more insertion, deletion, or point

mutation(s). Preferably, the isolated nucleic acid fragment encodes a mutant SAH hydrolase

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wherein the mutant SAH hydrolase is derived from a sequence of nucleotides set forth in SEQ
ID No. 1 and has one or more mutation selected from Phe 302 to Ser (F302S), Lys 186 to Ala
(K186A), His 301 to Asp (H301D), His 353 to Ser (H353S), Arg 343 to Ala (R343A), Asp 190
to Ala (D190A), Phe 82 to Ala (F82A), Thr 157 to Leu (T157L), Cys 195 to Asp (C195D),

5   Asn 181 to Asp (N181D), and deletion of Tyr 432 (Δ432). Also more preferably, the isolated
nucleic acid fragment encodes a mutant SAH hydrolase wherein the mutant SAH hydrolase is
derived from a sequence of nucleotides set forth in SEQ ID No. 1 and has a combination of
Arg 431 to Ala (R431A) and Lys 426 to Arg (K426R) mutations.

     Further provided is a plasmid, including the nucleic acid fragment encoding the above

10   mutant SAH hydrolases. Preferably, the plasmid is an expression vector including a sequence
of nucleotides encoding: a) a promoter region; and b) a mutant S-adenosylhomocysteine (SAH)
hydrolase, the mutant SAH hydrolase substantially retains its binding affinity or has enhanced
binding affinity for homocysteine (Hcy) or SAH but has attenuated catalytic activity. The
sequence of nucleotides encoding the mutant SAH hydrolase is operatively linked to the

15   promoter, whereby the mutant SAH hydrolase is expressed. More preferably, the plasmid also
includes a selectable marker.

     Further provided is a recombinant host cell containing the above plasmid. The
recombinant host cell can be any suitable host cell, including, but not limited to, a bacterial cell,
a yeast cell, a fungal cell, a plant cell, an insect cell or an animal cell.

20   Also provided are methods for producing a mutant SAH hydrolase. The recombinant
host cells can be grown or cultured under conditions whereby the mutant SAH hydrolase is
expressed by the cell. The expressed mutant SAH hydrolase can then be isolated or recovered.

     Additional mutant SAH hydrolase that substantially retains its binding affinity or has
enhanced binding affinity for homocysteine (Hcy) or SAH, but has attenuated catalytic activity

25   can be produced according to the procedures known to the those of skill in the art, including
procedures exemplified herein (see, *e.g.*, Section B). The above-described mutant SAH
hydrolases and additional mutant SAH hydrolase that substantially retain binding affinity or
have enhanced binding affinity for homocysteine (Hcy) or SAH but have attenuated catalytic
activity can be used for assaying Hcy in a sample.

30   **3.   Hcy assays using mutant SAH hydrolase**

     In one specific embodiment, the mutant Hcy-binding enzyme used in the Hcy assay is a
mutant SAH hydrolase, the mutant SAH hydrolase substantially retains its binding affinity or

has enhanced binding affinity for homocysteine (Hcy) or SAH but has attenuated catalytic activity. This assay, described in detail in the EXAMPLES, is depicted in Figure 1. In this Figure, the homocysteine-containing analyte is reduced to produce Hcy, which, is quantified or detected by binding it to a mutant (substrate trapping) SAH hydrolase; the Hcy is then

5 converted to SAH by reaction with adenosine in the presence of wild type SAH hydrolase. As exemplified in the Figure, instead of using a monoclonal antibody to effect quantitation (see, e.g., U.S. Patent No. 5,885,767 and U.S. Patent No. 5,631,127). Quantitation is effected using a fluorescence-labeled tracer S-adenosylcysteine in a competition binding format in which the mutant SAH is used to trap the substrate. Any suitable quantitation assay with any suitable

10 label can be used in the substrate trapping method. Figure 2 depicts an exemplary assay performed in a 96 well format; and figure 3 exemplifies preparation of labeling of adenosyl- cysteine with a fluorescent moiety.

In one preferred embodiment, the attenuated catalytic activity in the mutant SAH hydrolase is caused by mutation(s) in the mutant SAH hydrolase's binding site for NAD^+, or

15 mutation(s) in the mutant SAH hydrolase's catalytic site or a combination thereof.

In another preferred embodiment, the mutant SAH hydrolase has attenuated 5'- hydrolytic activity but substantially retains its 3'-oxidative activity.

In another preferred embodiment, the mutant SAH hydrolase irreversibly binds SAH.

In still another preferred embodiment, the mutant SAH hydrolase has a Km for SAH

20 that is about or less than 10.0 μM. More preferably, the mutant SAH hydrolase has a Km for SAH that is about 1.0 μM or less than 1.0 μM.

In yet another preferred embodiment, the mutant SAH hydrolase has a Kcat for SAH that is about or less than $0.1 \ S^{-1}$.

In yet another preferred embodiment, the mutant SAH hydrolase has one or more

25 insertion, deletion, or point mutation(s). More preferably, the mutant SAH hydrolase is derived from the sequence of amino acids set forth in SEQ ID No. 1 or encoded by the sequence of nucleotides set forth in SEQ ID No. 2 and has one or more of the following mutations: Phe 302 to Ser (F302S), Lys 186 to Ala (K186A), His 301 to Asp (H301D), His 353 to Ser (H353S), Arg 343 to Ala (R343A), Asp 190 to Ala (D190A), Phe 82 to Ala (F82A), Thr 157 to Leu

30 (T157L), Cys 195 to Asp (C195D), Asn 181 to Asp (N181D), and deletion of Tyr 432 (Δ432). Also more preferably, the mutant SAH hydrolase is derived from a sequence of amino acids set forth in SEQ ID No. 2 and has a combination of Arg 431 to Ala (R431A) and Lys 426 to Arg (K426R) mutations.

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In yet another preferred embodiment, prior to the contact between the sample and the mutant SAH hydrolase, oxidized Hcy in the sample is converted into reduced Hcy. More preferably, the oxidized Hcy in the sample is converted into reduced Hcy by a reducing agent such as tri-n-butylphosphine (TBP), ß-ME, DTT, dithioerythritol, thioglycolic acid,

5   glutathione, tris(2-carbxyethyl)phosphine, sodium cyanoborohydride, NaBH₄, KBH₄ and free metals.

In yet another preferred embodiment, prior to the contact between the sample and the mutant SAH hydrolase, the Hcy in the sample is converted into SAH. More preferably, the Hcy in the sample is converted into SAH by a wild-type SAH hydrolase. Also more preferably,

10   the SAH is contacted with the mutant SAH hydrolase in the presence of a SAH hydrolase catalysis inhibitor such as neplanocin A or thimersol.

In yet another preferred embodiment, prior to the contact between the SAH and the mutant SAH hydrolase, free adenosine is removed or degraded. More preferably, the free adenosine is degraded by combined effect of adenosine deaminase, purine nucleoside

15   phosphorylase and xanthine oxidase.

Any adenosine deaminase can be used. Preferably, the adenosine deaminase encoded by the nucleic acids having the following GenBank accession Nos. can be used: AF051275 (Caenorhabditis elegans); AI573492 (mouse mammary gland); AI462267 (mouse mammary gland); AI429519 (mouse embryo); AI429513 (mouse embryo); AI326688 (Mus musculus);

20   AI324114 (mouse placenta); AI322477 (mouse placenta); AI152550 (mouse uterus); U76422 (Human, SEQ ID No. 15; see also Lai, et al., Mol. Cell. Biol., 17(5):2413-24 (1997)); U76421 (Human); U76420 (Human); AI120695 (mouse mammary gland); AI049175 (Mus musculus); U73107 (Mus musculus); AF052506 (Mus musculus); AA871919 (Barstead bowel, Mus musculus); AA871917 (Barstead bowel, Mus musculus); AA871865 (Barstead bowel);

25   AA871752 (Barstead bowel); AA871702 (Barstead bowel); AA871324 (Barstead bowel); AA871189 (Barstead bowel); AA869711 (Mus musculus); ... ?69187 (Mus musculus); AA869184 (Mus musculus); AA869176 (Mus musculus); AA869120 (Mus musculus); U75503 (Homo sapiens); AA646698 (mouse mammary gland); AA646681 (mouse mammary gland); AA427106 (mouse mammary gland); D50624 (Streptomyces virginiae); AA389303 (mouse

30   embryo); AA389067 (mouse embryo); U88065 (Xenopus laevis); AA124740 (Mus musculus); U74586 (Rattus norvegicus); AA036487 (mouse placenta); AA035873 (mouse placenta); AA030290 (mouse placenta); AA023505 (mouse placenta); AA023331 (mouse placenta); AA111514 (mouse embryo); AA111327 (mouse embryo); AA110493 (mouse embryo);

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U73185 (Mus musculus); AA107590 (mouse embryo); AA102891 (mouse embryo);

AA097525 (mouse embryo); AA096642 (mouse embryo); AA087094 (mouse embryo);

AA060462 (mouse); U10439 (Human); M13792 (Human); U18942 (Rattus norvegicus);

K02567 (Human); M10319 (Mouse); M59033 (*E. coli* adenosine). Preferably, the adenosine

5   deaminase encoded by the nucleic acids having the following GenBank accession No. can be

used: U76422 (Human, SEQ ID No. 15; see also Lai, *et al.*, *Mol. Cell. Biol.*, 17(5):2413-24

(1997)).

Any purine nucleoside phosphorylase can be used. Preferably, the purine nucleoside

phosphorylase encoded by the nucleic acids having the following GenBank accession Nos. can

10   be used: U88529 (E.coli); U24438 (E.coli, SEQ ID No. 17; see also Cornell and Riscoe,

Biochim. Biophys. Acta, 1396(1):8-14 (1998)); U83703 (H. pyiori); and M30469 (*E. coli*).

Any xanthine oxidase can be used. Preferably, the xanthine oxidase encoded by the

nucleic acids having the following GenBank accession Nos. can be used: AF080548

(Sinorhizobium meliloti); and U39487 (Human, SEQ ID No. 19; see also Saksela and Raivio,

15   *Biochem. J.*, 315(1):235-9 (1996)).

In yet another preferred embodiment, the sample containing or suspected of containing

SAH is contacted with the mutant SAH hydrolase in the presence of a labeled SAH or a

derivative or an analog thereof, whereby the amount of the labeled SAH bound to the mutant

SAH hydrolase inversely relates to amount of the SAH in the sample. The SAH, or the

20   derivative or analog thereof, can be labeled by methods known in the art, *e.g.*, to become

radioactive, enzymatic, fluorescent, luminescent (including chemo- or bio-luminescent)

labeled. More preferably, the labeled SAH derivative or analog is a fluorescence labeled

adenosyl-cysteine.

In yet another preferred embodiment, the sample containing or suspected of containing

25   SAH is contacted with a labeled mutant SAH hydrolase. The mutant SAH hydrolase can be

labeled by methods known in the art, *e.g.*, to become radioactive, enzymatic, fluorescent,

luminescent (including chemo- or bio-luminescent) labeled. More preferably, the mutant SAH

hydrolase is fluorescently labeled. For cxample, a mutant SAH hydrolase derived from an

SAH hydrolase having sequence of amino acids encoded by the sequence of nucleotides set

30   forth in SEQ ID No. 2 is used and the mutant SAH hydrolase is fluorescently labeled at residue

Cys421.

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D. METHODS FOR ASSAYING FOLATE SPECIES

Further provided herein is a method for assaying a folate species in a sample. This method includes at least the steps of: a) contacting the sample with a mutant folate-species-binding enzyme, which substantially retains its binding affinity or has enhanced binding affinity for the folate species but has attenuated catalytic activity; and b) detecting binding between the folate species with the mutant folate-species-binding enzyme.

Any mutant folate-species-binding enzymes that substantially retain their binding affinity or have enhanced binding affinity for the folate species but have attenuated catalytic activity can be used in the folate species assay. Examples of such mutant folate-species-binding enzymes include mutant methionine synthase, tetrahydrofolate methyltransferase, methylenetetrahydrofolate reductase, folypolyglutamate synthase, dihydrofolate reductase and thymidylate synthase.

Nucleic acids encoding folate-species-binding enzymes can be obtained by methods known in the art. Where the molecules are available or the sequence known, they can be obtained from publicly available sources. Known nucleic acid sequences of folate-species-binding enzymes, such as methionine synthase, tetrahydrofolate methyltransferase, methylenetetrahydrofolate reductase, folypolyglutamate synthase, dihydrofolate reductase and thymidylate synthase, can be used in isolating nucleic acids encoding folate-species-binding enzymes from natural sources. Alternatively, nucleic acids encoding folate-species-binding enzymes can be obtained by chemical synthesis according to the known sequences.

In specific embodiment, the nucleotide sequences with the following GenBank accession Nos. can be used in obtaining nucleic acid encoding methionine synthase: AI547373 (Mesembryanthemum crystallinum); AI507856 (COBALAMINE-INDEPENDENT METHIONINE SYNTHASE); AI496185 (COBALAMINE-INDEPENDENT METHIONINE SYNTHASE); AI496016 (COBALAMINE-INDEPENDENT METHIONINE SYNTHASE); AI495904 (COBALAMINE-INDEPENDENT METHIONINE SYNTHASE); AI495702; AI495399; AI461276 (COBALAMINE-INDEPENDENT METHIONINE SYNTHASE); AI460827 (COBALAMINE-INDEPENDENT METHIONINE SYNTHASE); AI460549; AI443293; AI443243 (COBALAMINE-INDEPENDENT METHIONINE SYNTHASE); AI443242 (COBALAMINE-INDEPENDENT METHIONINE SYNTHASE); AI442736 (COBALAMINE-INDEPENDENT METHIONINE SYNTHASE); AI442546; AI442173 (COBALAMINE-INDEPENDENT METHIONINE SYNTHASE); AI442136

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(COBALAMINE-INDEPENDENT METHIONINE SYNTHASE); AI441314; AI440982; AI438053; AI416939 (COBALAMINE-INDEPENDENT METHIONINE SYNTHASE); AI416601; AI391967 (Conidial Neurospora crassa); AF034214 (Rattus norvegicus); U77388 (Chlamydomonas moewusii); AF093539 (Zea mays); U97200 (Arabidopsis thaliana); U36197

5   (Chlamydomonas reinhardtii); AF025794 (Homo sapiens); AJ222785 (Hordeum vulgare); Z49150 (C. blumei kinetoplast met gene); AB004651 (Hyphomicrobium methylovorum gene); AA661438 (Maize Leaf); AA661023 (Medicago truncatula); AA660965 (Medicago truncatula); AA660880 (Medicago truncatula); AA660780 (Medicago truncatula); AA660708 (Medicago truncatula); AA660643 (Medicago truncatula); AA660558 (Medicago truncatula);

10   AA660475 (Medicago truncatula); AA660444 (Medicago truncatula); AA660382 (Medicago truncatula); AA660310 (Medicago truncatula); AA660241 (Medicago truncatula); U75743 (Human); AA389835 (Arabidopsis thaliana); U84889 (Mesembryanthemum crystallinum); U73338 (Human); AA054818 (Maize Leaf); AA030695 (Maize Leaf); X83499 (C. roseus); U15099 (Saccharomyces cerevisiae (MET6)); J02804 (E. coli speED operon speE and speD

15   genes); M87625 (Escherichia coli); J04975 (E. coli). Preferably, the nucleotide sequences with the GenBank accession Nos. U75743 (SEQ ID No. 4) and U73338 (SEQ ID No. 6) can be used in obtaining nucleic acid encoding methionine synthase.

In another embodiment, the nucleotide sequences with the following GenBank accession Nos. can be used in obtaining nucleic acid encoding tetrahydrofolate

20   methyltransferase: Z99115 (SEQ ID No. 21; see also Kunst, et al., Nature, 390(6657):249-56 (1997)).

In still another specific embodiment, the nucleotide sequences with the following GenBank accession Nos. can be used in obtaining nucleic acid encoding methylenetetrahydrofolate reductase: AJ237672 (Homo sapiens); AH007491 (Mus musculus);

25   AF105998 (Mus musculus); AF105997 (Mus musculus); AF105996 (Mus musculus); AF105995 (Mus musculus); AF105994 (Mus musculus); AF105993 (Mus musculus); AF105992 (Mus musculus); AF105991 (Mus musculus); AF105990 (Mus musculus); AF105989 (Mus musculus); AF105988 (Mus musculus); AF102543 (Zymomonas mobilis); AH007464 (Homo sapiens complete CDs); AF105987 (Homo sapiens); AF105986 (Homo

30   sapiens); AF105985 (Homo sapiens); AF105984 (Homo sapiens); AF105983 (Homo sapiens); AF105982 (Homo sapiens); AF105981 (Homo sapiens); AF105980 (Homo sapiens); AF105979 (Homo sapiens); AF105978 (Homo sapiens); AF105977 (Homo sapiens); AI327505 (mouse); U74302 (Erwinia carotovora); AA660667 (Medicago truncatula); W11807 (mouse);

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AA368389 (Placenta I Homo sapiens); AA363389 (Ovary I Homo sapiens); U57049 (Rattus norvegicus); X07689 (X. typhimurium); and U09806 (Human). Preferably, the nucleotide sequences with the GenBank accession No. AH007464 can be used in obtaining nucleic acid encoding methylenetetrahydrofolate reductase (SEQ ID No. 23; see also Goyette, *et al.*, *Mamm.*

5   *Genome.*, 9(8):652-6 (1998)).

In yet another specific embodiment, the nucleotide sequences with the following GenBank accession Nos. can be used in obtaining nucleic acid encoding folypolyglutamate synthase: AL031852 (S. pombe); and M32445 (*E. coli*). Preferably, the nucleotide sequences with the GenBank accession No. M32445 can be used in obtaining nucleic acid encoding

10   folypolyglutamate synthase (SEQ ID No. 25; see also Bognar, *et al.*, *J. Biol. Chem.*, 262(25):12337-43 (1987)).

In yet another specific embodiment, the nucleotide sequences with the following GenBank accession Nos. can be used in obtaining nucleic acid encoding dihydrofolate reductase: AF083501 (Macaca mulatta rhadinovirus); AF028812 (Enterococcus faecalis);

15   U83347 (Kaposi's sarcoma-associated herpesvirus); U41366 (Cryptosporidium parvum); U03885 (Paramecium tetraurelia); AF006616 (Mycobacterium avium); U71365 (Kaposi's sarcoma-associated herpes-like virus fragment I); AF055727 (Streptococcus pneumoniae strain R6); AF055726 (Streptococcus pneumoniae strain AP183); AF055725 (Streptococcus pneumoniae strain AP13); AF055724 (Streptococcus pneumoniae strain AP173); AF055723

20   (Streptococcus pneumoniae strain AP92); AF055722 (Streptococcus pneumoniae strain AP71); AF055721 (Streptococcus pneumoniae strain AP188); AF055720 (Streptococcus pneumoniae strain AP48); AF077008 (Salmonella typhimurium plasmid pIE1142); AF073488 (Zea mays); M12742 (Coliphage T4); U84588 (Candida albicans); U12275 (Plasmodium berghei ANKA); U12338 (Pseudomonas aeruginosa); M18578 (S. cerevisiae); J03772 (Plasmodium falciparum);

25   L22484 (Trypanosoma cruzi); U09476 (Synthetic construct Tn7 (dhfr) gene); U31119 (Escherichia coli plasmid pDGO100); L08489 (Toxoplasma gondii); M69220 (*E. coli* plasmid pDGO100); L17041 (Synthetic construct); U40997 (Listeria monocytogenes); U20781 (Trypanosoma brucei); J01609 (*E. coli*); U43152 (Listeria monocytogenes); U36276 (Escherichia); U09273 (Shigella sonnei); M55264 (Herpesvirus saimiri); M20407 (Synthetic

30   mini type II); J05088 (H. volcanii); U10186 (Escherichia coli); M28071 (Herpesvirus saimiri); U12338 (Pseudomonas aeruginosa plasmid R1033); M18578 (S. cerevisiae); J03772 (Plasmodium falciparum (clone HB3)); L22484 (Trypanosoma cruzi); U09476 (Synthetic construct); U31119 (Escherichia coli plasmid pDGO100); L08489 (Toxoplasma gondii);

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M69220 (*E. coli* plasmid pDGO100); L17041 (Synthetic construct); U40997 (Listeria

monocytogenes); U20781 (Trypanosoma brucei); J01609 (*E. coli*); U43152 (Listeria

monocytogenes); U36276 (Escherichia coli); U09273 (Shigella sonnei); M55264 (Herpesvirus

saimiri); M20407 (Synthetic mini type II); J05088 (H. volcanii); U10186 (Escherichia coli);

5   M28071 (Herpesvirus saimiri); M19237 (Herpesvirus saimiri); L26316 (Mus musculus);

L15311 (Cricetulus sp.); M37124 (Chinese hamster); M19869 (Chinese hamster); M26668

(Saccharomyces cerevisiae); M26496 (Pneumocystis carinii); M26495 (P. carinii); L08594

(Arabidopsis thaliana); L08593 (Arabidopsis thaliana); K01804 (Bacteriophage T4); M22852

(C. fasciculata); M30834 (P. chabaudi); J04643 (P. falciparum); J03028 (P. falciparum);

10   M22159 (P. falciparum); M14330 (L. tropica); M12734 (Leishmania); K02118 (Plasmid R67

from *E. coli*); J03306 (Plasmid pAZ1 type III); M10922 (Lactobacillus casei); M26022

(Enterobacter aerogenes); M84522 (Escherichia coli); M26023 (Citrobacter freundii); and

U06861 (Drosophila melanogaster). Preferably, the nucleotide sequences with the GenBank

accession No. M37124 can be used in obtaining nucleic acid encoding dihydrofolate reductase

15   (SEQ ID No. 27; see also Dicker, *et al.*, *J. Biol. Chem.*, 265(14):8317-21 (1990)).

In yet another specific embodiment, the nucleotide sequences with the following

GenBank accession Nos. can be used in obtaining nucleic acid encoding thymidylate synthase:

AF083501 (Macaca mulatta rhadinovirus, thymidylate synthase); AF059506 (chilo iridescent

virus); AI531067 (Drosophila melanogaster Schneider L2 cell); AI515689 (LD Drosophila

20   melanogaster embryo; AI514354 (Drosophila melanogaster embryo; AB023402 (Oryza sativa

thyA); AI406263 (Drosophila melanogaster head; AI390061 (Drosophila melanogaster head;

AF099673 (Caenorhabditis elegans); AF099672 (Ascaris suum); AI297939 (Drosophila

melanogaster larval-early pupal); AI293665 (Drosophila melanogaster larval-early pupal);

AI136006 (Drosophila melanogaster head); AI258021 (Drosophila melanogaster larval-early

25   pupal); D00596 (Homo sapiens); AF029302 (Rhesus monkey rhadinovirus); U83348 (Kaposi's

sarcoma-associated herpesvirus); U69259 (Synthetic Plasmodium falciparum); U12256

(Filobasidiella neoformans); U41366 (Cryptosporidium parvum); U03885 (Paramecium

tetraurelia); U86637 (Neisseria gonorrhoeae); U71365 (Kaposi's sarcoma-associated herpes-

like virus); AF073994 (Drosophila melanogaster); AF073488 (Zea mays); M12742 (Coliphage

30   T4); U12275 (Plasmodium berghei ANKA); J03772 (Plasmodium falciparum (clone HB3);

L22484 (Trypanosoma cruzi); L08489 (Toxoplasma gondii); L12138 (Rattus); U20781

(Trypanosoma brucei); M29019 (Synthetic Lactobacillus); L31962 (Bacteriophage beta-22);

M13190 (Herpesvirus saimiri); M14080 (Herpesvirus saimiri); M22036 (Herpesvirus ateles);

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M13019 (Mouse); M30774 (Mouse); J04230 (C.albicans); L08594 (Arabidopsis thaliana);

L08593 (Arabidopsis thaliana); K01804 (Bacteriophage T4); M30834 (P.chabaudi); J04643 (P.

falciparum); J03028 (P.falciparum); M14330 (L.tropica); M12734 (Leishmania); M19653

(L.casei (thyA)); and M33770 (L.lactis (thyA)). Preferably, the nucleotide sequences with the

5   GenBank accession No. D00596 can be used in obtaining nucleic acid encoding thymidylate

synthase (SEQ ID No. 29; see also Kaneda, *et al., J. Biol. Chem.*, 265(33):20277-84 (1990)).

Once nucleic acids encoding folate-species-binding enzymes are obtained, these nucleic

acids can be mutagenized and screened and/or selected for folate-species-binding enzymes that

substantially retain their binding affinity or have enhanced binding affinity for the folate

10 species but have attenuated catalytic activity. Insertion, deletion, or point mutation(s) can be

introduced into nucleic acids encoding folate-species-binding enzymes according to the

methods described in Section B.

Information regarding the structural-functional relationship of the folate-species-

binding enzymes can be used in the mutagenesis and selection of the folate-species-binding

15 enzymes that substantially retain their binding affinity or have enhanced binding affinity for the

folate species but have attenuated catalytic activity. For example, mutants can be made in the

enzyme's binding site for its co-enzyme, co-factor, a non-folate-species substrate, or in the

mutant enzyme's catalytic site, or a combination thereof.

In one specific embodiment, the folate species is 5,-methyl-tetrahydrofolate, the mutant

20 folate-species-binding enzyme is a mutant methionine synthase, and the attenuated catalytic

activity of the mutant methionine synthase is caused by mutation in its catalytic site, its binding

site for vitamin B_{12}, Hcy, or a combination thereof.

In another specific embodiment, the folate species is tetrahydrofolate, the mutant folate-

species-binding enzyme is a mutant tetrahydrofolate methyltransferase, and the attenuated

25 catalytic activity of the mutant tetrahydrofolate methyltransferase is caused by mutation in its

catalytic site, its binding site for serine, or a combination thereof.

In still another specific embodiment, the folate species is 5, 10,-methylene

tetrahydrofolate, the mutant folate-species-binding enzyme is a mutant

methylenetetrahydrofolate reductase, and the attenuated catalytic activity of the

30 methylenetetrahydrofolate reductase is caused by mutation in its catalytic site.

In yet another specific embodiment, the folate species is 5, 10,-methylene

tetrahydrofolate, the mutant folate-species-binding enzyme is a mutant folypolyglutamate

synthase, and the attenuated catalytic activity of the folypolyglutamate synthase is caused by

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mutation in its catalytic site, its binding site for ATP, L-glutamate, Mg^{2+}, a combination thereof.

In yet another specific embodiment, the folate species is dihydrofolate, the mutant folate-species-binding enzyme is a mutant dihydrofolate reductase, and the attenuated catalytic

5   activity of the mutant dihydrofolate reductase is caused by mutation in its catalytic site, its binding site for NADPH, or a combination thereof. Preferably, the mutant dihydrofolate reductase is a *Lactobacillus casei* dihydrofolate reductase having an Arg43Ala or Trp21His mutation (Basran, *et al.*, *Protein Eng.*, 10(7):815-26 91997)).

In yet another specific embodiment, the folate species is 5, 10,-methylene

10   tetrahydrofolate, the mutant folate-species-binding enzyme is a mutant thymidylate synthase, and the attenuated catalytic activity of the mutant thymidylate synthase is caused by mutation in its catalytic site, its binding site for dUMP, or a combination thereof. Preferably, the mutant thymidylate synthase is a human thymidylate synthase having a mutation selected from Tyr6His, Glu214Ser, Ser216Ala, Ser216Leu, Asn229Ala and His199X, X being any amino

15   acid that is not His (Schiffer, *et al.*, *Biochemistry*, 34(50):16279-87 (1995); Steadman, *et al.*, *Biochemistry*, 37:7089-7095 (1998); Williams, *et al.*, *Biochemistry*, 37(20):7096-102 (1998); Finer-Moore, *et al.*, *J. Mol. Biol.*, 276(1):113-29 (1998); and Finer-Moore, *et al.*, *Biochemistry*, 35(16):5125-36 (1996)). Also more preferably, the mutant thymidylate synthase is an *E. coli* thymidylate synthase having an Arg126Glu mutation (Strop, *et al.*, *Protein Sci.*, 6(12):2504-11

20   (1997)) or a *Lactobacillus casei* thymidylate synthase having a V316Am mutation (Carreras, *et al.*, *Biochemistry*, 31(26):6038-44 (1992)).

Once a mutant folate-species-binding enzyme with desired properties, *i.e.*, substantially retaining its binding affinity or having enhanced binding affinity for the folate species but has attenuated catalytic activity, is identified, such mutant folate-species-binding enzyme can be

25   produced by any methods known in the art including recombinant expression, chemical synthesis or a combination thereof as described in Section B. Preferably, the mutant folate-species-binding enzyme is obtained by recombinant expression.

E.   METHODS FOR ASSAYING CHOLESTEROL

Further provided herein is a method for assaying cholesterol in a sample. This method

30   includes at least the steps of: a) contacting the sample with a mutant cholesterol-binding enzyme, the mutant enzyme substantially retains its binding affinity or has enhanced binding

affinity for cholesterol but has attenuated catalytic activity; and b) detecting binding between cholesterol with the mutant cholesterol-binding enzyme.

Any mutant cholesterol-binding enzymes that substantially retain their binding affinity or have enhanced binding affinity for cholesterol but have attenuated catalytic activity can be

5   used in the cholesterol assay. Examples of such mutant cholesterol-binding enzymes include mutant cholesterol esterase and cholesterol oxidase.

Cholesterol-binding enzymes

Nucleic acids encoding cholesterol-binding enzymes can be obtained by methods known in the art or obtained from public or commercial sources. Known nucleic acid

10 sequences of cholesterol-binding enzymes, such as cholesterol esterase and cholesterol oxidase, can be used in isolating nucleic acids encoding cholesterol-binding enzymes from natural sources. Alternatively, nucleic acids encoding cholesterol-binding enzymes can be obtained by chemical synthesis according to the known sequences.

In one embodiment, the nucleotide sequences with the following GenBank accession

15 Nos. can be used in obtaining nucleic acid encoding cholesterol esterase: AI558069 (Mouse mammary gland); AI465062 (Mouse mammary gland); AA793597 (Mouse diaphragm); AA762311 (Mouse mammary gland); AA759540 (Mouse mammary gland); AA672047 (Mouse mammary gland); AA571290 (Mouse diaphragm); AA537778 (Mouse diaphragm); AA265434 (Mouse); M69157 (Rat pancreatic); U33169 (Mus musculus); L46791 (Rattus

20 norvegicus); M85201 (Human). Preferably, the nucleotide sequences with the GenBank accession Nos. M85201 (SEQ ID No. 31), nucleotide sequences described in U.S. Patent No. 5,624,836 (bovine pancreatic cholesterol esterase; SEQ ID No. 33) can be used in obtaining nucleic acid encoding cholesterol esterase.

In another specific embodiment, the nucleotide sequences with the following GenBank

25 accession Nos. can be used in obtaining nucleic acid encoding cholesterol oxidase: E07692; E07691; E03850 (Brevibacterium sterolicum); E03828; E03827; D00712 (B. sterolicum choB gene); U13981 (Streptomyces A19249 choM gene); and M31939 (Streptomyces A19249 choP gene). Preferably, the nucleotide sequences with the GenBank accession No. U13981 (SEQ ID No. 35; see also Corbin, *et al.*, *Appl. Environ. Microbiol.*, 60(12):4239-44 (1994)) and the

30 nucleotide sequence described in U.S. Patent No. 5,665,560 (SEQ ID No. 37) can be used in obtaining nucleic acid encoding cholesterol oxidase.

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Once nucleic acids encoding cholesterol-binding enzymes are obtained, these nucleic acids can be mutagenized and screened and/or selected for cholesterol-binding enzymes that substantially retain their binding affinity or have enhanced binding affinity for cholesterol but have attenuated catalytic activity. Insertion, deletion, or point mutation(s) can be introduced

5   into nucleic acids encoding cholesterol-species-binding enzymes according to the methods described in Section B.

Information regarding the structural-functional relationship oi uie cholesterol-binding enzymes can be used in the mutagenesis and selection of the cholesterol-binding enzymes that substantially retain their binding affinity or have enhanced binding affinity for cholesterol but

10   have attenuated catalytic activity. For example, mutants can be made in the enzyme's binding site for its co-enzyme, co-factor, a non-cholesterol substrate, or in the mutant enzyme's catalytic site, or a combination thereof.

In one specific embodiment, the mutant cholesterol-binding enzyme is a mutant cholesterol esterase, and the attenuated catalytic activity of the mutant cholesterol esterase is

15   caused by mutation in its catalytic site, its binding site for H_2O or a combination thereof. Preferably, the cholesterol esterase is a pancreatic cholesterol esterase having a Ser194Thr or Ser194Ala mutation (DiPersio, *et al.*, *J. Biol. Chem.*, 265(28):16801-6 (1990)).

In another specific embodiment, the mutant cholesterol-binding enzyme is a mutant cholesterol oxidase, and the attenuated catalytic activity of the mutant cholesterol oxidase is

20   caused by mutation in its catalytic site, its binding site for O_2 or a combination thereof. Preferably, the cholesterol oxidase is a *Brevibacterium sterolicum* cholesterol oxidase having a His447Asn or His447Gln mutation (Yue, *et al.*, *Biochemistry*, 38(14):4277-86 (1999)).

Once a mutant cholesterol-binding enzyme with desired properties, *i.e.*, substantially retaining its binding affinity or having enhanced binding affinity for the cholesterol but has

25   attenuated catalytic activity, is identified, such mutant cholesterol-binding enzyme can be produced by any methods known in the art including recombinant expression, chemical synthesis or a combination thereof as described in Section B. Preferably, the mutant cholesterol-binding enzyme is obtained by recombinant expression.

F.   HCY ASSAYS IN CONJUNCTION WITH CHOLESTEROL AND/OR FOLIC
30   ACID ASSAY

The Hcy assays described in Section C can be conducted in conjunction with a cholesterol and/or a folic acid assay.

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1.   Cholesterol assay

Cholesterol assay can be conducted according to any methods known in the art. For example, the Hcy assays described in Section C can be conducted in conjunction with cholesterol assays described in Section E. In addition, the Hcy assays can be conducted in
5   conjunction with cholesterol assays described in U.S. Patent Nos. 4,161,425, 4,164,448, 4,188,188, 4,211,531, 5,034,332, 5,047,327, 5,217,873 and 5,593,894.

U.S. Patent No. 4,161,425 describes cholesterol assay enzymatic reagents for rate determination of cholesterol in a sample to be assayed. The reagents contain cholesterol oxidase, and a buffering agent in an amount to produce a solution having a pH of between
10   about 5.5 and about 8. The reagent acts by neutralizing substantially all oxygen consumption inhibiting effects of inhibiting agents present in the sample to be assayed, such as an alkyldimethylbenzylammonium salt in an amount sufficient to neutralize substantially all oxygen consumption inhibiting effects of inhibiting agents present in the sample to be assayed. U.S. Patent No. 4,161,425 also describes methods for determining the cholesterol concentration
15   in a cholesterol containing sample by: (a) oxidizing the cholesterol present in the sample in an oxygen saturated aqueous solution by means of a cholesterol assay enzymatic reagent;
(b) generating a first electrical signal related to the oxygen concentration; (c) differentiating the first electrical signal to produce an output signal proportional to the instantaneous time rate of change of oxygen concentration; and (d) measuring the output signal to determine the
20   cholesterol concentration. In this method substantially all oxygen consumption inhibiting effects of inhibiting agents in the sample to be assayed is neutralized by including in the cholesterol assay enzymatic reagent a cationic surfactant in an amount sufficient to neutralize substantially all oxygen consumption inhibiting effects of inhibiting agents present in the sample to be assayed, preferably, from about 0.01 to about 0.4 percent by weight of the reagent
25   of a cationic surfactant. The enzymatic agent is cholesterol oxidase and a buffering agent in an amount to produce a solution having a pH of between 5.5 and about 8; in the presence of a sensor which serves to monitor a property or characteristic of oxygen in the solution related to the oxygen concentration thereof;

U.S. Patent No. 4,164,448 describes diagnostic agents in solid form for the detection
30   and determination of cholesterol and cholesterol esters in body fluids. The agents include a solid carrier having impregnated or embedded therein cholesterol oxidase, a system for the detection of hydrogen peroxide, buffer and from 2 to 30%, based on the total solid diagnostic

agent of at least one surface-active compound with lipophilic and hydrophilic properties. U.S. Patent No. 4,164,448 also describes processes for the activation of analytically pure, detergent-free, storage-stable cholesterol oxidase, recovered from a micro-organism by extraction with a surfactant, for the analytic determination of cholesterol. The processes include removing all traces of the surfactant from the cholesterol oxidase to produce a surfactant-free cholesterol oxidase and then adding to an aqueous solution of the surfactant-free cholesterol oxidase between 0.005% to 0.1% by weight, based on the weight of the aqueous cholesterol oxidase solution, of at least one surface-active compound with lipophilic and hydrophilic properties before use of the cholesterol oxidase.

U.S. Patent No. 4,188,188 describes compositions for use in a HDL cholesterol separation. The compositions contain heparin, a divalent cation salt having the formula: CX_2, where C is selected from Group IIA metals and manganese and X is a halogen, and an inert filler that includes a polysaccharide, a terminal interlocking linear glucose polymer and a vinylpyrrolidone polymer. This patent also describes high density lipoprotein cholesterol assays utilizing heparin/$MnCl_2$ precipitation. In these assays the serum sample to be assayed is added to a reagent composition as described above. The resulting supernatant is assayed for cholesterol.

U.S. Patent No. 4,211,531 describes methods of determining cholesterol in a biological sample. The methods include a precipitation step for precipitating protein in the sample, a color forming step for forming in the resulting supernatant a color proportional to the concentration of at least one form of cholesterol in the sample, and a step of determining the depth of color formed. The precipitation step is carried out by means of a reagent that contains colorimetric amounts of propionic acid and ferric ion. U.S. Patent No. 4,211,531 also describes methods of determining cholesterol in a biological sample using a color forming step in which a reaction mixture including at least a fraction of the serum and a color forming reagent is formed. The depth of color formed is related to the amount of at least one form of cholesterol in the reaction mixture. In these assays, the reaction mixture contains a colorimetric amount of sulfuric acid and propionic acid. U.S. Patent No. 4,211,531 also describes methods of determining cholesterol in a sample of human serum, by first precipitating protein in the sample by means of a protein precipitation reagent that contains colorimetric amounts of propionic acid and ferric ion to produce a generally protein-free supernatant. Color is then developed in a reaction mixture containing the supernatant and a cholesterol color reagent, which contains colorimetric amounts of propionic acid and sulfuric acid. The depth of color formed is related

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to the amount of cholesterol in the sample. U.S. Patent No. 4,211,531 also provides reagent kits for determination of total cholesterol, which include a first container containing a colorimetric amount of ferric chloride and propionic acid and a second container containing a reagent that contains colorimetric amount of propionic acid and sulfuric acid.

5   U.S. Patent No. 5,034,332 describes assays for the presence of HDL cholesterol in a blood plasma sample. This method includes the steps of: mixing the sample with a proteinaceous material that is also present in protein H of boar vesicle seminal plasma so as to cause a precipitation of HDL cholesterol bound to the proteinaceous material; and measuring either the amount of cholesterol in a supernatant formed by the mixing step, or the amount of

10   precipitant formed in the mixing step.

         U.S. Patent No. 5,217,873 describes stable cholesterol assay compositions that contain: (a) at least one acidic compound selected from a bile acid and a salt of a bile acid, the total of the acid compound being present in an amount of up to about 5 mM; (b) a nonionic surfactant present in a concentration of from about 0.15 to about 1.5 percent by volume; (c) a buffer in a

15   concentration of from 0 to about v (d) cholesterol oxidase in a concentration of at least about 0.02 KIU/l; (e) cholesterol esterase present in a concentration of at least about 0.07 KIU/l; and (f) a chromogen system for determination of hydrogen peroxide, the cholesterol assay solution having a pH of from about 5.5 to about 7.5 and a completion time of less than 10 minutes at 37°C. U.S. Patent No. 5,217,873 also describes stable total cholesterol chromogen assay

20   compositions containing an aqueous solution have a pH of from about 6.5 to about 7.5 and (a) phenol in a concentration of from about 8 to about 35 mM; (b) a metal salt of cholic acid present in a concentration of from about 0.2 to about 5 mM; (c) a nonionic surfactant present in a concentration of from about 0.2 to about 1.5 percent volume by volume; (d) a phosphate buffer present in a concentration of from about 0.5 to about 30 mM and sufficient to maintain a

25   pH of from about 6 to about 7.5; (e) 4-aminoantipyrine in a concentration up to about 0.3 mM; (f) cholesterol esterase present in a concentration of at least about 0.07 KIU/l; (g) cholesterol oxidase present in a concentration of at least about 0.02 KIU/l; and (h) peroxidase, the amount of peroxidase and 4-aminoantipyrine being sufficient to enable quantitative determination of the amount of hydrogen peroxide formed from oxidation of cholesterol within 10 minutes at

30   37°C. U.S. Patent No. 5,217,873 further describes stable total cholesterol chromogen assay compositions containing an aqueous solution of: a) phenol in a concentration of about 17 mM; b) 2,4dichlorophenol present in a concentration of about 0.5 mM; c) a metal salt of cholic acid

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present in a concentration of up to about 5 mM; d) polyethylene glycol p-isooctylphenyl ether

present in a concentration of from about 0.2 to about 0.6 percent volume by volume; e)

KH_2PO_4 present in a concentration of about 12.5 mM; f) peroxidase present in a concentration

of about 30 KIU/l; g) cholesterol oxidase present in a concentration of at least about 0.05

5   KIU/l; h) microbial cholesterol esterase present in a concentration of at least about 0.1 KIU/l;

and i) 4-aminoantipyrene present in concentration of about 0.3 mM, the stable total cholesterol

chromogen assay composition having a pH of from about 6.0 to about 7.5.

U.S. Patent No. 5,593,894 describes methods for forming a spectrophotometrically

active product of cholesterol, such as HDL-C, LDL-C and VLDL-C. The method includes

10   contacting cholesterol with an acyl compound and a perchlorate effective to form a

spectrophotometrically active product of the cholesterol, the perchlorate selected from zinc

perchlorate, barium perchlorate and perchloric acid. U.S. Patent No. 5,593,894 also describes

methods for determining the amount of cholesterol present in a test sample by contacting a test

sample in which cholesterol is present with an acyl compound and a perchlorate effective to

15   form a spectrophotometrically active product with the cholesterol, the perchlorate selected from

zinc perchlorate, barium perchlorate and perchloric acid, and evaluating the spectrophotometric

activity to determine the amount of the cholesterol present in the sample.

U.S. Patent No. 5,047,327 describes stable cholesterol assay compositions. These

compositions contain a polyhydroxy compound free aqueous solution of: (a) at least one acidic

20   compound selected from a bile acid and a salt of a bile acid, the total of the acidic compound

being present in a positive amount of up to about 5 mM; (b) a nonionic surfactant present in a

concentration of from about 0.15 to about 1.5 percent volume by volume; (c) a buffer in a

concentration of from 0 to about 65 mM; (d) cholesterol oxidase in a concentration of at least

about 0.02 KIU/l, (e) microbial cholesterol esterase in a concentration of at least about 0.07

25   KIU/l; and (f) a chromogen system for determination of hydrogen peroxide; the cholesterol

assay solution having a pH of from about 5.5 to about 8.5 a stability of at least 3 days at 41°C

and an assay completion time within 10 minutes at 37°C. U.S. Patent No. 5,047,327 also

describes stable total cholesterol chromagen assay compositions. These compositions are

aqueous solutions having a pH of from about 6.5 to about 7.5 and (a) phenol in a concentration

30   of from about 8 to about 35 mM; (b) sodium cholate present in a concentration of from about

0.2 to about 5 mM; (c) a nonionic surfactant present in a concentration of from about 0.15 to

about 1.5 percent volume by volume; (d) a buffer present in a concentration of from 0.5 to

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about 65 mM; (e) 4-aminoantipyrine; (f) microbial cholesterol esterase present in a

concentration of at least about 0.07 KIU/l; (g) cholesterol oxidase present in a concentration of

at least about 0.02 KIU/l; and (h) peroxidase, the amount of peridase and 4-aminoantipyrine

being sufficient to enable quantitative determination of the amount of hydrogen peroxide

5   formed from oxidation of cholesterol within 10 minutes at 37.degree. C., the assay composition

having a stability of at least 3 days at 41°C.

2.   Folic acid assay

Folic acid assay can be conducted according to any methods known in the art. For

example, the Hcy assays described in Section C can be conducted in conjunction with folic acid

10   assays described in Section D. In addition, the Hcy assays can be conducted in conjunction

with cholesterol assays described in U.S. Patent Nos. 4,276,280, 4,336,185, 4,337,339,

5,374,560 and 5,800,979.

U.S. Patent No. 4,276,280 describes derivatives of folic acid wherein the α-carboxyl

group of the glutamyl moiety is substituted with a radical which is capable of being

15   radioiodinated, such as, substituted and unsubstituted tyrosyl and histidyl. The radioiodinated

derivatives can be employed as tracers for the assay of folates.

U.S. Patent No. 4,336,185 describes protein conjugates and iodinated conjugates of

folic acid and its salts, esters and amides which retain the ability to competitively bind on a

binding protein, such as folic acid binding globulin or on an antibody which is specific to folic

20   acid. The compounds are useful in analysis of body fluids such as blood serum, blood plasma,

urine and the like, to assay for the presence of folic acid by competitive protein binding assay

(CPSA) or by radioimmunoassay (RIA) procedures.

U.S. Patent No. 4,337,339 describes that folic acid derivatives, such as radiolabeled

pteroyltyrosine, are conveniently synthesized from either pteroic acid or by the direct

25   condensation of 6-formylpterin with p-aminobenzoyltyrosine methyl ester. The radioiodinated

derivatives are particularly useful in competitive protein binding and radioimmuno-assays of

folate compounds.

U.S. Patent No. 5,374,560 describes methods for detecting a deficiency of cobalamin or

folic acid in warm-blooded animals, by: assaying a body fluid for an elevated level of

30   cystathionine; and correlating an elevated level of cystathionine in the body fluid with a

likelihood of a deficiency of cobalamin or folic acid. U.S. Patent No. 5,374,560 also describes

methods for detecting a deficiency of cobalamin in warm-blooded animals, by: assaying a body

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fluid for an elevated level of 2-methylcitric acid I or 2-methylcitric acid II or; and correlating
an elevated level of 2-methylcitric acid I or 2-methylcitric acid II or in the body fluid with a
likelihood of a deficiency of cobalamin. U.S. Patent No. 5,374,560 further describes methods
for detecting a deficiency of cobalamin or folic acid in warm-blooded animals and for

5   distinguishing between a deficiency of cobalamin and a deficiency of folic acid, by: assaying a
first body fluid from the warm-blooded animal for an elevated level of cystathionine;
correlating an elevated level of cystathionine in the body fluid with a likelihood of a deficiency
of cobalamin or folic acid; assaying a second body fluid from the warm-blooded animal having
an elevated level of cystathionine in the first body fluid correlating with a likelihood of a

10   deficiency of cobalamin or folic acid, for an elevated level of 2-methylcitric acid I or 2-
methylcitric acid II or; and correlating an elevated level of 2-methylcitric acid I or 2-
methylcitric acid II or in the second body fluid with a likelihood of a deficiency of cobalamin
but a likelihood of a deficiency of folic acid. U.S. Patent No. 5,374,560 further describes
methods for detecting a deficiency of cobalamin or folic acid in warm-blooded animals, by:

15   assaying a first body fluid for an elevated level of cystathionine; assaying a second body fluid
for an elevated level of homocysteine; and correlating an elevated level of cystathionine and
homocysteine with a likelihood of a deficiency of cobalamin or folic acid.

         U.S. Patent No. 5,800,979 describes methods for determination of concentration in a
body fluid of at least one member of an endogenous folate co-enzyme pool selected from: (1)

20   pool I containing tetrahydrofolate, dihydrofolate and 5,10-methylenetetrahydrofolate; (2) pool
II containing 5-methyltetrahydrofolate; and (3) pool III containing 3-formyltetrahydrofolate,
10-formyltetrahydrofolate, 5,10-methyleneyltetrahydrofolate, and 5-formiminotetrahydrofolate.
The method includes the steps of: (a) combining a known amount of at least one internal
standard folate co-enzyme which is a non-radioactively-labeled stable isotope of a member of

25   the selected folate co-enzyme pool with the body fluid, wherein the internal standard folate
coenzyme is recovered from harvested bacterial cells grown on a medium containing non-
radioactively-labeled stable isotope paraaminobenzoic acid; (b) at least partially purifying the
endogenous and internal standard folate coenzymes from other components in the body fluid in
a partial purification step; (c) quantitating the endogenous folate co-enzymes in the purified

30   body fluid of step (b) by gas chromatography/mass spectrometry analysis; and (d) determining
the concentration of the selected endogenous folate coenzyme pool by correcting the
concentrations of endogenous folate coenzymes quantitated in step (c) for endogenous losses as
reflected by losses in the known amount of internal standard folate co-enzyme of step (a).

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G.   METHODS FOR ASSAYING BILE ACID AND BILE SALTS

Further provided herein is a method for assaying bile acids or bile salts in a sample by:
a) contacting the sample with a mutant bile-acid-binding enzyme or bile-salt-binding enzyme,
the mutant enzyme substantially retains its binding affinity or has enhanced binding affinity for
5   the bile acid or bile salt but has attenuated catalytic activity; and b) detecting binding between
the bile acid or bile salt with the mutant bile-acid-binding enzyme or bile-salt-binding enzyme.

Any mutant bile-acid-binding enzyme or bile-salt-binding enzyme that substantially
retain their binding affinity or have enhanced binding affinity for the bile acid or bile salt but
have attenuated catalytic activity can be used in the bile acid or bile salt assay. Example of
10   such mutant bile-acid-binding enzyme or bile-salt-binding enzyme includes 3-α-hydroxy
steroid dehydrogenase.

Nucleic acids encoding bile-acid-binding enzymes or bile-salt-binding enzymes can be
obtained by methods known in the art. Known nucleic acid sequences of bile-acid-binding
enzyme or bile-salt-binding enzyme, such as 3-α-hydroxy steroid dehydrogenase, can be used
15   in isolating nucleic acids encoding bile-acid-binding enzymes or bile-salt-binding enzymes
from natural sources. Alternatively, nucleic acids encoding bile-acid-binding enzymes or bile-
salt-binding enzymes can be obtained by chemical synthesis according to the known sequences.

In one specific embodiment, the nucleotide sequences with the following GenBank
accession Nos. can be used in obtaining nucleic acid encoding 3-α-hydroxy steroid
20   dehydrogenase: AA866404 (Rattus norvegicus); AA866403 (Rattus norvegicus); U34072 (Mus
musculus); AF064635 (Mus musculus putative steroid); AB009304 (Anas platyrhynchos);
D17310 (Rat); U32426 (Molluscum contagiosum virus); L23428 (Comamonas testosteroni);
M67467 (Macaca fuscata); M27137 (Human). Preferably, the nucleotide sequences with the
GenBank accession No. M27137 (SEQ ID No. 39; see also The, *et al.*, *Mol. Endocrinol.*,
25   3(8):1310-2 (1989)) can be used in obtaining nucleic acid encoding 3-α-hydroxy steroid
dehydrogenase.

Once nucleic acids encoding bile-acid-binding enzymes or bile-salt-binding enzymes
are obtained, these nucleic acids can be mutagenized and screened and/or selected for bile-acid-
binding enzymes or bile-salt-binding enzymes that substantially retain their binding affinity or
30   have enhanced binding affinity for bile acids or bile salts but have attenuated catalytic activity.
Insertion, deletion, or point mutation(s) can be introduced into nucleic acids encoding bile-acid-
binding enzymes or bile-salt-binding enzymes according to the methods described in Section B.

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Information regarding the structural-functional relationship of the bile-acid-binding enzymes or bile-salt-binding enzymes can be used in the mutagenesis and selection of the bile-acid-binding enzymes or bile-salt-binding enzymes that substantially retain their binding affinity or have enhanced binding affinity for bile acids or bile salts but have attenuated

5   catalytic activity. For example, mutants can be made in the enzyme's binding site for its co-enzyme or for a non-bile-acid or non-bile-salt substrate, or in the mutant enzyme's catalytic site, or a combination thereof.

In one specific embodiment, the mutant bile-acid-binding enzyme is a mutant 3-α-hydroxy steroid dehydrogenase, and the attenuated catalytic activity of the mutant 3-α-hydroxy

10   steroid dehydrogenase is caused by mutation in its catalytic site, its binding site for NAD^+ or a combination thereof.

Once a mutant bile-acid-binding enzyme or bile-salt-binding enzyme with desired properties, i.e., substantially retaining its binding affinity or having enhanced binding affinity for the bile acid or bile salt but having attenuated catalytic activity, is identified, such mutant

15   bile-acid-binding enzyme or bile-salt-binding enzyme can be produced by any methods known in the art including recombinant expression, chemical synthesis or a combination thereof as described in Section B. Preferably, the mutant bile-acid-binding enzyme or bile-salt-binding enzyme is obtained by recombinant expression.

H.   METHODS FOR ASSAYING GLUCOSE

20   Further provided herein is a method for assaying glucose in a sample. This method includes at least the steps of: a) contacting the sample with a mutant glucose-binding enzyme, the mutant enzyme substantially retains its binding affinity or has enhanced binding affinity for glucose but has attenuated catalytic activity; and b) detecting binding between glucose with the mutant glucose-binding enzyme.

25   Any mutant glucose-binding enzymes that substantially retain their binding affinity or have enhanced binding affinity for glucose but have attenuated catalytic activity can be used in the glucose assay. Examples of such mutant glucose-binding enzyme include mutant glucose isomerase, glucokinase, hexokinase and glucose oxidase.

Nucleic acids encoding glucose-binding enzymes can be obtained by methods known in

30   the art. Known nucleic acid sequences of glucose-binding enzymes, such as glucose isomerase, glucokinase, hexokinase and glucose oxidase, can be used in isolating nucleic acids encoding

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glucose-binding enzymes from natural sources. Alternatively, nucleic acids encoding glucose-binding enzymes can be obtained by chemical synthesis according to the known sequences.

In one specific embodiment, the nucleotide sequences with the following GenBank accession Nos. can be used in obtaining nucleic acid encoding glucose isomerase: AF065160

5 (Toxoplasma gondii); AF050755 (Giardia intestinalis (GPI2)); AF050754 (Giardia intestinalis (GPI1)); AI117811 (mouse mammary gland); AA636682 (mouse myotubes); AA611494 (mouse irradiated colon); AA529061 (mouse irradiated colon); AA522284 (mouse embryonic region); AA472600 (mouse mammary gland); L27675 (Drosophila yakuba isofemale line 4); D13777 (Synechocystis sp.); AA265107 (mouse pooled organs); AA162075 (mouse skin);

10 AA139952 (mouse heart); AA117013 (mouse embryonic region); W36773 (mouse); W16112 (mouse); AA03546 (mouse embryo); W77098 (mouse embryo); W61997 (mouse embryo); W53620 (mouse embryo); U17225 (Zea mays); L27685 (Drosophila yakuba isofemale line 1); L27684 (Drosophila yakuba isofemale line 13); L27683 (Drosophila yakuba isofemale line 12); L27682 (Drosophila yakuba isofemale line 11); L27681 (Drosophila yakuba isofemale line 10);

15 L27680 (Drosophila yakuba isofemale line 9); L27679 (Drosophila yakuba isofemale line 8); L27678 (Drosophila yakuba isofemale line 7); L27677 (Drosophila yakuba isofemale line 6); L27676 (Drosophila yakuba isofemale line 5); L27555 (Drosophila melanogaster isochromosomal line); L27554 (Drosophila melanogaster isochromosomal line); L27553 (Drosophila melanogaster isochromosomal line); L27674 (Drosophila yakuba isofemale line 3);

20 and L27673 (Drosophila yakuba isofemale). Preferably, the nucleotide sequences with the GenBank accession No. U17225 (SEQ ID No. 41; see also Lal and Sachs, et al., *Plant Physiol.*, 108(3):1295-6 (1995)) can be used in obtaining nucleic acid encoding glucose isomerase.

In another specific embodiment, the nucleotide sequences with the following GenBank accession Nos. can be used in obtaining nucleic acid encoding glucokinase: AI386017 (Mouse

25 testis); AI325384 (Mouse embryo); AI323376 (Mouse embryo); AI255715 (Mouse liver mlia); AI196901 (Mouse liver); AI194797 (Mouse liver); AI194643 (Mouse liver); U44834 (Mycobacterium tuberculosis); U21919 (Brucella abortus); L41631 (Mus musculus); AI035808 (Mouse kidney); AI035659 (Mouse liver); AA882226 (Mouse lung); AH005826 (Homo sapiens pancreatic beta cell specific glucokinase (GCK) and major liver specific glucokinase

30 (GCK) genes); AF041022 (Homo sapiens glucokinase); M69051 (Human liver glucokinase (ATP:D-hexose 6-phosphotransferase); AA109998 (Mouse testis); AA014441 (Mouse embryo); L38990 (Mus musculus); U22490 (Escherichia coli); M24077 (Saccharomyces cerevisiae); M90299 (Human); M88011 (Human pancreatic beta-cell); M25807 (Rat); J04218

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(Rat); M60615 (Z.mobilis). Preferably, the nucleotide sequences with the GenBank accession No. M90299 (SEQ ID No. 43; see also Koranyi, et al., Diabetes, 41(7):807-11 (1992)) can be used in obtaining nucleic acid encoding glucokinase.

In still another specific embodiment, the nucleotide sequences with the following
5   GenBank accession Nos. can be used in obtaining nucleic acid encoding glucose oxidase: AF012277 (Penicillium amagasakiense); U56240 (Talaromyces flavus); X16061 (Aspergillus niger gox gene); X56443 (A.niger god gene); J05242 (A.niger); AF012277 (Penicillium amagasakiense); U56240 (Talaromyces flavus); X16061 (Aspergillus niger gox gene); X56443 (A.niger god gene); J05242 (A.niger glucose). Preferably, the nucleotide sequences with the
10 GenBank accession No. J05242 (SEQ ID No. 45; see also Frederick, et al., J. Biol. Chem., 265(7):3793-802 (1990)) and the nucleotide sequences described in U.S. Patent No. 5,879,921 (SEQ ID No. 47) can be used in obtaining nucleic acid encoding glucose oxidase.

Once nucleic acids encoding glucose-binding enzymes are obtained, these nucleic acids can be mutagenized and screened and/or selected for glucose-binding enzymes that
15 substantially retain their binding affinity or have enhanced binding affinity for glucose but have attenuated catalytic activity. Insertion, deletion, or point mutation(s) can be introduced into nucleic acids encoding glucose-binding enzymes according to the methods described in Section B.

Information regarding the structural-functional relationship of the glucose-binding
20 enzymes can be used in the mutagenesis and selection of the glucose-binding enzymes that substantially retain their binding affinity or have enhanced binding affinity for glucose but have attenuated catalytic activity. For example, mutants can be made in the enzyme's binding site for its co-enzyme, co-factor, non-glucose substrate, or in the mutant enzyme's catalytic site, or a combination thereof.

25 In one specific embodiment, the mutant glucose-binding enzyme is a Clostridium thermosulfurogenes glucose isomerase having a mutation selected from His101Phe, His101Glu, His101Gln, His101Asp and His101Asn (Lee, et al., J. Biol. Chem., 265(31):19082-90 (1990)). In another specific embodiment, the mutant glucose-binding enzyme is a mutant hexokinase or glucokinase, and the attenuated catalytic activity of the mutant hexokinase or
30 glucokinase is caused by mutation in its catalytic site, its binding site for ATP or Mg^{2+}, or a combination thereof. In still another specific embodiment, the mutant glucose-binding enzyme is a mutant glucose kinase, and the attenuated catalytic activity of the mutant glucose kinase is caused by mutation in its catalytic site, its binding site for H_2O or O_2, or a combination thereof.

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Once a mutant glucose-binding enzyme with desired properties, *i.e.*, substantially retaining its binding affinity or having enhanced binding affinity for glucose but has attenuated catalytic activity, is identified, such mutant glucose-binding enzyme can be produced by any methods known in the art including recombinant expression, chemical synthesis or a

5   combination thereof as described in Section B. Preferably, the mutant glucose-binding enzyme is obtained by recombinant expression.

I.   METHODS FOR ASSAYING ETHANOL

Further provided herein is a method for assaying ethanol in a sample. This method includes at least the steps of: a) contacting the sample with a mutant ethanol-binding enzyme,

10   the mutant enzyme substantially retains its binding affinity or has enhanced binding affinity for ethanol but has attenuated catalytic activity; and b) detecting binding between ethanol with the mutant ethanol-binding enzyme.

Any mutant ethanol-binding enzymes that substantially retain their binding affinity or have enhanced binding affinity for ethanol but have attenuated catalytic activity can be used in

15   the ethanol assay. Examples of such mutant ethanol-binding enzyme include alcohol dehydrogenase.

Nucleic acids encoding ethanol-binding enzymes can be obtained by methods known in the art. Known nucleic acid sequences of ethanol-binding enzymes, such as alcohol dehydrogenase, can be used in isolating nucleic acids encoding ethanol-binding enzymes from

20   natural sources. Alternatively, nucleic acids encoding ethanol-binding enzymes can be obtained by chemical synthesis according to the known sequences.

In one specific embodiment, the nucleotide sequences with the following GenBank accession Nos. can be used for producing mutant nucleic acid molecules encoding alcohol dehydrogenase: AI194923 (mouse liver); U16293 (Human class IV); U73514 (Human short-

25   chain); U09623 (Human); M30471 (Human class III); Z21104 (Human adult Testis tissue); L33179 (Human class IV sigma-1); M24317 (Human class I); M29872 (Human); M81118 (Human); M21692 (Human class I); M12963 (Human class I); M68895 (Human); U07821 (Human); AF044127 (Homo sapiens peroxisomal short-chain); M12272 (Homo sapiens); D00137 (Homo sapiens); L47166 (Homo sapiens); M12271 (Homo sapiens class I); Z21104

30   (Human adult Testis tissue). In addition, nucleic acid molecules, such as those provided in the following U.S. Patents can be used in obtaining and producing mutant nucleic acid encoding alcohol dehydrogenase:

U.S. Patent No.	alcohol dehydrogenase
5,908,924	thermoanaerobacter ethanolicus 39E secondary-alcohol dehydrogenase
5,855,881	Mammalian alcohol dehydrogenase
5,385,833	Pseudomonas sp. ATCC No. 49794 alcohol dehydrogenase
5,344,777	membrane-bound alcohol dehydrogenase complex
5,342,767	Lactobacillus kefir alcohol dehydrogenase 5,225,339
5,162,516	alcohol dehydrogenase II gene from Zymomonas mobilis

Nucleic acid molecules that include the sequences of sequences with the GenBank accession Nos. U73514 (SEQ ID No. 49), U09623 (SEQ ID No. 51; see also Kedishvili, *et al.*, *J. Biol. Chem.*, 270(8):3625-30 (1995)), M30471 (SEQ ID No. 53; see also Sharma, *et al.*,

5   *Biochem. Biophys. Res. Commun.*, 164(2):631-7 (1989)) and M24317 (SEQ ID No. 55; see also Xu, *et al.*, *Genomics*, 2(3):209-14 (1988); Ikuta, *et al.*, *Proc. Natl. Acad. Sci.*, 82(9):2703-7 (1985)) can be used in obtaining nucleic acid encoding alcohol dehydrogenase.

Once nucleic acids encoding ethanol-binding enzymes are obtained, these nucleic acids can be mutagenized and screened and/or selected for ethanol-binding enzymes that

10   substantially retain their binding affinity or have enhanced binding affinity for ethanol but have attenuated catalytic activity. Insertion, deletion, or point mutation(s) can be introduced into nucleic acids encoding ethanol-binding enzymes according to the methods described in Section B.

Information regarding the structural-functional relationship of the ethanol-binding

15   enzymes can be used in the mutagenesis and selection of the ethanol-binding enzymes that substantially retain their binding affinity or have enhanced binding affinity for ethanol but have attenuated catalytic activity. For example, mutants can be made in the enzyme's binding site for its co-enzyme, co-factor, non-ethanol substrate, or in the mutant enzyme's catalytic site, or a combination thereof.

20   In one specific embodiment, the mutant ethanol-binding enzyme is a mutant alcohol dehydrogenase and the attenuated catalytic activity of the mutant alcohol dehydrogenase is caused by mutation in its catalytic site, its binding site for NAD^+ or Zn^{2+}, or a combination thereof. Preferably, the mutant alcohol dehydrogenase is a human liver alcohol dehydrogenase having a His51Gln mutation (Ehrig, *et al.*, *Biochemistry*, 30(4):1062-8 (1991)). Also

25   preferably, the mutant alcohol dehydrogenase is a horse liver alcohol dehydrogenase having a

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Phe93Trp or Val203Ala mutation (Bahnson, *et al., Proc. Natl. Acad. Sci.,* 94(24):12797-802 (1997); Colby, *et al., Biochemistry,* 37(26):9295-304 (1998)).

Once a mutant ethanol-binding enzyme with desired properties, *i.e.,* substantially retaining its binding affinity or having enhanced binding affinity for ethanol but having

5   attenuated catalytic activity, is identified, such mutant ethanol-binding enzyme can be produced by any methods known in the art including recombinant expression, chemical synthesis or a combination thereof as described in Section B. Preferably, the mutant ethanol-binding enzyme is obtained by recombinant expression.

J.   METHODS FOR ASSAYING URIC ACID

10   Further provided herein is a method for assaying uric acid in a sample. This method includes at least the steps of: a) contacting the sample with a mutant uric-acid-binding enzyme, the mutant enzyme substantially retains its binding affinity or has enhanced binding affinity for uric acid but has attenuated catalytic activity; and b) detecting binding between uric acid with the mutant uric-acid-binding enzyme.

15   Any mutant uric-acid-binding enzymes that substantially retain their binding affinity or have enhanced binding affinity for uric acid but have attenuated catalytic activity can be used in the uric acid assay. Examples of such mutant uric acid-binding enzyme include urate oxidase or uricase.

Nucleic acids encoding uric-acid-binding enzymes can be obtained by methods known

20   in the art. Known nucleic acid sequences of uric-acid-binding enzymes, such as urate oxidase or uricase, can be used in isolating nucleic acids encoding uric-acid-binding enzymes from natural sources. Alternatively, nucleic acids encoding uric-acid-binding enzymes can be obtained by chemical synthesis according to the known sequences.

In one specific embodiment, the nucleotide sequences with the following GenBank

25   accession Nos. can be used in obtaining nucleic acid encoding urate oxidase or uricase: AB028150 (Medicago sativa); AB028149 (Medicago sativa); E13225 (Arthrobacter globiformis); U72663 (Phaseolus vulgaris); D86930; D86929; D32043 (Candida utilis); D49974 (Bacillus sp.); M10594 (Soybean nodulin-35 (N-35)); M24396 (Rat); M27695 (Mouse); M27694 (Baboon); and M27697 (Pig). In addition, the nucleotide sequences

30   described in the following U.S. Patent Nos. can be used in obtaining nucleic acid encoding urate oxidase or uricase: 5,541,098 (SEQ ID No. 57) and 5,728,562 (SEQ ID No. 59). Preferably, the nucleotide sequences with the GenBank accession No. M27694 (SEQ ID

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No. 61; see also Wu, *et al.*, *Proc. Natl. Acad. Sci.*, 86(23):9412-6 (1989)) can be used in

obtaining nucleic acid encoding urate oxidase or uricase.

Once nucleic acids encoding uric-acid-binding enzymes are obtained, these nucleic

acids can be mutagenized and screened and/or selected for uric-acid-binding enzymes that

5   substantially retain their binding affinity or have enhanced binding affinity for uric acid but

have attenuated catalytic activity. Insertion, deletion, or point mutation(s) can be introduced

into nucleic acids encoding uric-acid-binding enzymes according to the methods described in

Section B.

Information regarding to structural-functional relationship of the uric-acid-binding

10   enzymes can be used in the mutagenesis and selection of the uric-acid-binding enzymes that

substantially retain their binding affinity or have enhanced binding affinity for uric acid but

have attenuated catalytic activity. For example, mutants can be made in the enzyme's binding

site for its co-enzyme, co-factor, non-uric-acid substrate, or in the mutant enzyme's catalytic

site, or a combination thereof.

15   In one specific embodiment, the mutant uric-acid-binding enzyme is a mutant urate

oxidase or uricase, and the attenuated catalytic activity of the mutant urate oxidase or uricase is

caused by mutation in its catalytic site, its binding site for O_2, H_2O, or copper ion, or a

combination thereof. Preferably, the mutant urate oxidase is a rat urate oxidase having a

mutation selected from H127Y, H129Y and F131S (Chu, *et al.*, *Ann. N.Y. Acad. Sci.*, 804:781-6

20   (1996)).

Once a mutant uric acid binding enzyme with desired properties, *i.e.*, substantially

retaining its binding affinity or having enhanced binding affinity for uric acid but having

attenuated catalytic activity, is identified, such mutant uric-acid-binding enzyme can be

produced by any methods known in the art including recombinant expression, chemical

25   synthesis or a combination thereof as described in Section B. Preferably, the mutant uric-acid-

binding enzyme is obtained by recombinant expression.

K.   OTHER PROGNOSTIC AND DIAGNOSTIC ASSAYS AND ASSAYS FOR MONITORING THERAPEUTIC INTERVENTION

1.   Diagnostic and prognostic assays

30   Small molecule markers associated with various diseases, defects or conditions can be

monitored for diagnostics and prognostics. The presence, absence or quantitation of any

diagnostic and prognostic small molecule markers can be monitored, and a diagnostic or

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prognostic determination can be made based on the assay results. The following Table 2
illustrates exemplary markers for certain diseases or conditions and mutant enzymes to be used
in the substrate trapping assay methods.

Table 2

Metabolites	Enzymes	Diseases
Bile acid	3α-hydroxysteroid dehydrogenase	biliary cirrhosis
Uric acid	Uricase	gout, leukemia
Creatinine	creatinine amidohydrolase	renal disfunction
Serotonin	serotonin N-acetyltransferase	neuron disease
Hyaluronic acid	hyaluronidase	rheumatoid arthritis
Catecholamine	catechol O-methyltransferase	neuroblastoma
Homovanillic acid	monoamine oxidase	neuroblastoma
Vanilylmandelic acid	dopamine beta-hydroxylase	neuroblastoma

5   In a specific embodiment, the small molecule analyte to be assayed is bile acid and the
mutant analyte-binding enzyme is a mutant 3α-hydroxysteroid dehydrogenase, the mutant 3α-
hydroxysteroid dehydrogenase substantially retains its binding affinity or has enhanced binding
affinity for bile acid but has attenuated catalytic activity. Preferably, the attenuated catalytic
10   activity is caused by mutation in the mutant 3α-hydroxysteroid dehydrogenase's catalytic site,
its binding site for NADP$^+$, or a combination thereof.

In another specific embodiment, the small molecule analyte to be assayed is creatinine
and the mutant analyte-binding enzyme is a mutant creatinine amidohydrolase, the mutant
creatinine amidohydrolase substantially retains its binding affinity or has enhanced binding
15   affinity for creatinine but has attenuated catalytic activity. Preferably, the attenuated catalytic
activity is caused by mutation in the mutant creatinine amidohydrolase's catalytic site, its
binding site for H_2O, or a combination thereof.

In still another specific embodiment, the small molecule analyte to be assayed is
serotonin and the mutant analyte-binding enzyme is a mutant serotonin N-acetyltransferase, the
20   mutant serotonin N-acetyltransferase substantially retains its binding affinity or has enhanced
binding affinity for serotonin but has attenuated catalytic activity. Preferably, the attenuated

catalytic activity is caused by mutation in the mutant serotonin N-acetyltransferase's catalytic site, its binding site for AcCoA, or a combination thereof.

In yet another specific embodiment, the small molecule analyte to be assayed is hyaluronic acid and the mutant analyte-binding enzyme is a mutant hyaluronidase, the mutant

5   hyaluronidase substantially retains its binding affinity or has enhanced binding affinity for hyaluronic acid but has attenuated catalytic activity. Preferably, the attenuated catalytic activity is caused by mutation in the mutant hyaluronidase's catalytic :··, ·. binding site for H_2O, or a combination thereof.

In yet another specific embodiment, the small molecule analyte to be assayed is

10   catecholamine and the mutant analyte-binding enzyme is a mutant catechol O-methyltransferase, the mutant catechol O-methyltransferase substantially retains its binding affinity or has enhanced binding affinity for catecholamine but has attenuated catalytic activity. Preferably, the attenuated catalytic activity is caused by mutation in the mutant catechol O-methyltransferase's catalytic site, its binding site for AdoMet or Mg^{2+}, or a combination thereof.

15   In yet another specific embodiment, the small molecule analyte to be assayed is homovanillic acid and the mutant analyte-binding enzyme is a mutant monoamine oxidase, the mutant monoamine oxidase substantially retains its binding affinity or has enhanced binding affinity for homovanillic acid but has attenuated catalytic activity. Preferably, the attenuated catalytic activity is caused by mutation in the mutant monoamine oxidase's catalytic site, its

20   binding site for O_2, or a combination thereof.

In yet another specific embodiment, the small molecule analyte to be assayed is vanilylmandelic acid and the mutant analyte-binding enzyme is a mutant dopamine ß-hydroxylase, the mutant dopamine ß-hydroxylase substantially retains its binding affinity or has enhanced binding affinity for vanilylmandelic acid but has attenuated catalytic activity.

25   Preferably, the attenuated catalytic activity is caused by mutation in the mutant dopamine ß-hydroxylase's catalytic site, its binding site for O_2 or ascorbic acid, or a combination thereof.

More preferably, the mutant dopamine ß-hydroxylase is a mammalian enzyme, such as the bovine dopamine beta-hydroxylase having one or more mutations that correspond to mutations of the bovine enzyme at Tyr477, His249 or Arg503 (Robertson, *et al.*, *J. Biol.*

30   *Chem.*, 265:1029-1035 (1990); and Farrington, *et al.*, *J. Biol. Chem.*, 265(2):1036-40 (1990)); or a dopamine ß-hydroxylase having one or more mutations at its copper binding site (Blackburn, *et al.*, *Biochemistry*, 27(16):6001-8 (1988); or a dopamine ß-hydroxylase having one or more mutations within a region of the enzyme corresponding to the sequence (SEQ ID

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No. 154): Ala-Pro-Asp-Val-Leu-Ile-Pro-Gly-Gln-Gln-Thr-Thr-Tyc-Trp-Cys-Tyr-Val-Thr-Glu-Leu-Pro-Asp-Gly-Phe-Pro-Arg, where Tyc is an amino acid residue with the in-chain mass of a cresol-Tyr adduct (106 + 163 Da) (see, e.g., DeWolf, et al., Biochemistry, 27(26):9093-101 (1988) of the bovine enzyme.

5   2.   Drug assays

The substrate trapping methods provided herein can also be used for monitoring presence, absence, quantitation, kinetics and metabolism of therapeutic or preventive agents, especially small molecule agents. Assays for any drug or therapeutic agent for which an enzyme that binds to the drug or agent can be identified are contemplated herein. The

10   following Table 3 illustrates exemplary therapeutic or preventive agents that can be assayed and the target enzymes for modifying for use in the assays provided herein.

Table 3

Drug	Enzyme	Therapeutic target
Cyclosporin A	calcineurine/cyclophilin	immunosuppressant
Mycophenoric acid	inosine monophosphate dehydrogenase	immunosuppressant
Leflunomide	dihydroorotate dehydrogenase	immunosuppressant
N-acetylprocainamide	procainamide N-acetyltransferase	cardiac arrhythmias
Fluvastatin	HMG-CoA reductase	hypercholesterolemia
Lovastatin	HMG-CoA reductase	hypercholesterolemia
Provastatin	HMG-CoA reductase	hypercholesterolemia
Simvastatin	HMG-CoA reductase	hypercholesterolemia
Atorvastain	HMG-CoA reductase	hypercholesterolemia
Finasteride	S2-reductase	benign prostate hyperplasia

Thus, if the small molecule analyte to be assayed is cyclosporin A, the mutant analyte-

15   binding enzyme is a mutant calcineurine or cyclophilin, that has been designed to substantially retain its binding affinity or have enhanced binding affinity for cyclosporin A but have attenuated catalytic activity. Preferably, the attenuated catalytic activity is achieved by a mutation in calcineurine's catalytic site, its binding site for Ca^{2+} and/or calmodulin, or a combination thereof. More preferably, mutant calcineurine to be used is the bovine brain

20   calcineurin containing mutations in its Fe^{3+}-Zn^{2+} binding site (see, Yu, et al., Biochemistry,

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36(35):10727-34 (1997)); and the mutant cyclophilin to be used is the human cyclophilin A
having one or more of the following mutations:. W121A, H54Q, R55A, F60A, Q111A, F113A,
and H126Q (Liu, et al., Biochemistry, 30:2306-2310 (1991); and Zydowsky, et al., Protein Sci.,
1(9):1092-9 (1992)).

5   In another specific embodiment, the small molecule analyte to be assayed is
mycophenoric acid and the mutant analyte-binding enzyme is a mutant inosine monophosphate
dehydrogenase (IMPDH), the mutant inosine monophosphate dehydrogenase substantially
retains its binding affinity or has enhanced binding affinity for mycophenoric acid but has
attenuated catalytic activity. Preferably, the attenuated catalytic activity is caused by mutation

10   in the mutant inosine monophosphate dehydrogenase's catalytic site, its binding site for NAD^+,
or a combination thereof. More preferably, the mutant IMPDH is the human type II isoform of
IMPDH having mutation(s) at Cys 331 (Colby, et al., Proc. Natl. Acad. Sci., 96(7):3531-6
(1999)).

        In still another specific embodiment, the small molecule analyte to be assayed is
15   leflunomide and the mutant analyte-binding enzyme is a mutant dihydroorotate dehydrogenase,
the mutant dihydroorotate dehydrogenase substantially retains its binding affinity or has
enhanced binding affinity for leflunomide but has attenuated catalytic activity. Preferably, the
attenuated catalytic activity is caused by mutation in the mutant dihydroorotate
dehydrogenase's catalytic site, its binding site for O_2, or a combination thereof. More

20   preferably, the mutant dihydroorotate dehydrogenase is the Lactococcus lactis dihydroorotate
dehydrogenase having one or more of the mutations: C130S, C130A, K43A, K43E, N132A and
K164A (Bjornberg, et al., Biochemistry, 36(51):16197-205 (1997); or the E.coli dihydroorotate
dehydrogenase having the S175C mutation (Bjornberg, et al., Biochemistry, 38(10):2899-908
(1999)); or the Lactococcus lactis dihydroorotate dehydrogenase having one or more mutations

25   at the following locations: Asn 67, Asn 127, Asn 132, Asn 193, Lys 43, Ser 194, Met 69,
Gly 70 and Leu 71 (Rowland, et al., Protein Sci., 7(6):1269-79 (1998)).

        In yet another specific embodiment, the small molecule analyte to be assayed is N-
acetylprocainamide and the mutant analyte-binding enzyme is a mutant procainamide N-
acetyltransferase, the mutant procainamide N-acetyltransferase substantially retains its binding

30   affinity or has enhanced binding affinity for N-acetylprocainamide but has attenuated catalytic
activity. Preferably, the attenuated catalytic activity is caused by mutation in the mutant
procainamide N-acetyltransferase's catalytic site, its binding site for acetyl CoA, or a
combination thereof.

In yet another specific embodiment, the small molecule analyte to be assayed is selected
from fluvastatin, lovastatin, provastatin, simvastatin and atorvastatin and the mutant analyte-
binding enzyme is a mutant HMG-CoA reductase (hydroxymethylglutaryl-CoA reductase), the
mutant HMG-CoA reductase substantially retains its binding affinity or has enhanced binding
5   affinity for N-fluvastatin, lovastatin, provastatin, simvastatin or atorvastatin, but has attenuated
catalytic activity. Preferably, the attenuated catalytic activity is caused by mutation in the
mutant HMG-CoA reductase's catalytic site, its binding site for NADPH, or a combination
thereof. More preferably, the mutant HMG-CoA reductase is the *Pseudomonas mevalonii*
HMG-CoA reductase having one or more of the following mutations: K267A, K267H, K267R,
10 or having one or more mutations at His 381 (Bochar, *et al.*, *Biochemistry*, 38(28):8879-83
(1999); Tabernero, *et al.*, *Proc. Natl. Acad. Sci.*, 96(13):7167-71 (1999); or the *Syrian hamster*
HMG-CoA reductase having one or more of the following mutations: E558D, E558Q, D766N
and phosphorylated Ser 871 (Omkumar and Rodwell, *J. Biol. Chem.*, 269(24):16862-6 (1994);
and Frimpong and Rodwell, *J. Biol. Chem.*, 269(2):1217-21 (1994)).

15 **L.   COMBINATIONS, KITS AND ARTICLES OF MANUFACTURE**

Combinations and kits containing such combination are provided. The combination
includes: a) a mutant analyte-binding enzyme, the mutant enzyme substantially retains its
binding affinity or has enhanced binding affinity for the analyte or an immediate analyte
enzymatic conversion product but has attenuated catalytic activity; and b) reagents and/or other
20 means for detecting binding between the analyte or the immediate analyte enzymatic
conversion product and the mutant analyte-binding enzyme. Preferably, the analyte to be
assayed is Hcy. Also preferably, binding between the Hcy or the immediate Hcy enzymatic
conversion product and the mutant Hcy-binding enzyme is detected using a labelled Hcy, a
labelled immediate Hcy enzymatic conversion product, a labelled mutant Hcy-binding enzyme,
25 or a derivative or an analog thereof. More preferably, wherein the analyte to be assayed is Hcy,
the combination also includes reagents for detecting cholesterol and/or folic acid. The kit can
also include instructions for assaying an analyte in a sample using the mutant analyte binding
enzymes.

The packages discussed herein in relation to diagnostic systems are those customarily
30 utilized in diagnostic systems. Such packages include glass and plastic, such as polyethylene,
polypropylene and polycarbonate, bottles and vials, plastic and plastic-foil laminated

envelopes and the like.   The packages may also include containers appropriate for use in auto

analyzers. The packages typically include instructions for performing the assays.

In still another embodiment, an article of manufacture is provided. The article includes:

a) packaging material; b) a mutant analyte-binding enzyme, the mutant enzyme substantially

5   retains its binding affinity or has enhanced binding affinity for the analyte or an immediate

analyte enzymatic conversion product but has attenuated catalytic activity; and c) a label

indicating that the mutant analyte-binding enzyme and the reagents are for use in assaying the

analyte in a sample. The article of manufacture may also include reagents for detecting binding

between the analyte or the immediate analyte enzymatic conversion product and the mutant

10   analyte-binding enzyme.

M.   PREPARATION OF CONJUGATES

Conjugates, such as fusion proteins and chemical conjugates, of the mutant analyte-

binding enzyme with a protein or peptide fragment (or plurality thereof) that functions, for

example, to facilitate affinity isolation or purification of the mutant enzyme, attachment of the

15   mutant enzyme to a surface, or detection of the mutant enzyme are provided. The conjugates

can be produced by chemical conjugation, such as via thiol linkages, but are preferably

produced by recombinant means as fusion proteins. In the fusion protein, the peptide or

fragment thereof is linked to either the N-terminus or C-terminus of the mutant enzyme. In

chemical conjugates the peptide or fragment thereof may be linked anywhere that conjugation

20   can be effected, and there may be a plurality of such peptides or fragments linked to a single

mutant enzyme or to a plurality thereof.

1.   Conjugation

Conjugation can be effected by any method known to those of skill in the art. As

described below, conjugation can be effected by chemical means, through covalent, ionic or

25   any other suitable linkage.

a.   Fusion proteins

Fusion proteins are provided herein. A fusion protein contains: a) one or a plurality of

mutant analyte-binding enzymes and b) at least one protein or peptide fragment that facilitates,

for example: i) affinity isolation or purification of the fusion protein; ii) attachment of the

30   fusion protein to a surface; or iii) detection of the fusion protein, or any combination thereof.

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The facilitating agent is selected to perform the desired purpose, such as (i) - (iii), and is linked a mutant analyte-binding enzyme such that the resulting conjugate retains the analyte binding property and also pocesses the property(ies) of the facilitating agent. For example, the facilitating agent can be a protein or peptide fragment, such as a protein binding peptide,

5   including but not limited to an epitope tag or an IgG binding protein, a nucleotide binding protein, such as a DNA or RNA binding protein, a lipid binding protein, a polysaccharide binding protein, and a metal binding protein or fragments thereof that possess the requisite desired facilitating activity.

Such facilitating agents can be designed, screened or selected according to the methods

10   known in the art. The screening or selection process begins, for example, with nucleic acid encoding a particular protein or peptide to be used in the fusion protein, and screened or selected for its specific binding partner. Alternatively, the screening or selection process can start with a specific molecule that can be used in the subsequent isolation/purification, attachment or detection, and screen or select for a particular protein or peptide sequence to be

15   used in the fusion protein that can specifically bind to the pre-selected molecule.

The conventional technique of random screening of natural products can be used in screening and selecting a protein or peptide sequence and its specific binding partner. In addition, numerous strategies can be used for preparing proteins having new binding specificities. These new approaches generally involve the synthetic production of large

20   numbers of random molecules followed by some selection procedure to identify the molecule of interest. For example, epitope libraries have been developed using random polypeptides displayed on the surface of filamentous phage particles. The library is made by synthesizing a repertoire of random oligonucleotides to generate all combinations, followed by their insertion into a phage vector. Each of the sequences is separately cloned and expressed in phage, and the

25   relevant expressed peptide can be selected by finding those phage that bind to the particular target. The phages recovered in this way can be amplified and the selection repeated. The sequence of the peptide is decoded by sequencing the DNA (See, *e.g.*, Cwirla, *et al.*, *Proc. Natl. Acad. Sci., USA*, 87:6378-6382 (1990); Scott, *et al.*, *Science*, 249:386-390 (1990); and Devlin, *et al.*, *Science*, 249:404-406 (1990).

30   Another approach involves large arrays of peptides that are synthesized in parallel and screened with acceptor molecules labelled with fluorescent or other reporter groups. The sequence of any effective peptide can be decoded from its address in the array (See, *e.g.*,

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Geysen, *et al., Proc. Natl. Acad. Sci., USA,* 81:3998-4002 (1984); Maeji, *et al., J. Immunol. Met.,* 146:83-90 (1992); and Fodor, *et al., Science,* 251:767-775 (1991).

    Combinatorial approaches can also be employed. For example, in one exemplary approach, combinatorial libraries of peptides are synthesized on resin beads such that each resin

5   bead contains about 20 pmoles of the same peptide. The beads are screened with labeled acceptor molecules and those with bound acceptor are searched for by visual inspection, physically removed, and the peptide identified by direct sequence analysis (Lam, *et al., Nature,* 354:82-84 (1991)). Another useful combinatory method for identification of peptides of desired activity is that of Houghten, *et al.* (see, *e.g.,, Nature,* 354:84-86 (1991)). For

10   hexapeptides of the 20 natural amino acids, 400 separate libraries are synthesized, each with the first two amino acids fixed and the remaining four positions occupied by all possible combinations. An assay, based on competition for binding or other activity, is then used to find the library with an active peptide. Twenty new libraries are then synthesized and assayed to determine the effective amino acid in the third position, and the process is reiterated in this

15   fashion until the active hexapeptide is defined.

b.   Chemical conjugation

    To effect chemical conjugation herein, the targeting agent is linked via one or more selected linkers or directly to the targeted agent. Chemical conjugation must be used if the targeted agent is other than a peptide or protein, such a nucleic acid or a non-peptide drug.

20   Any means known to those of skill in the art for chemically conjugating selected moieties may be used.

1.   Heterobifunctional cross-linking reagents

    Numerous heterobifunctional cross-linking reagents that are used to form covalent bonds between amino groups and thiol groups and to introduce thiol groups into proteins, are

25   known to those of skill in this art (see, *e.g.,* the PIERCE CATALOG, ImmunoTechnology Catalog & Handbook, 1992-1993, which describes the preparation of and use of such reagents and provides a commercial source for such reagents; see, also, *e.g.,* Cumber, *et al.* (1992) *Bioconjugate Chem.* 3`:397-401; Thorpe, *et al.* (1987) *Cancer Res.* 47:5924-5931; Gordon, *et al.* (1987) *Proc. Natl. Acad Sci.* 84:308-312; Walden, *et al.* (1986) *J. Mol. Cell Immunol.*

30   2:191-197; Carlsson, *et al.* (1978) *Biochem. J. 173:* 723-737; Mahan, *et al.* (1987) *Anal. Biochem. 162:*163-170; Wawryznaczak, *et al.* (1992) *Br. J. Cancer 66:*361-366; Fattom, *et al.*

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(1992) *Infection & Immun.* 60:584-589). These reagents may be used to form covalent bonds

between the the mutant analyte binding enzyme and the facilitating agent. These reagents

include, but are not limited to: N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP; disulfide

linker); sulfosuccinimidyl 6-[3-(2-pyridyldithio)propionamido]hexanoate (sulfo-LC-SPDP);

5   succinimidyloxycarbonyl-α-methyl benzyl thiosulfate (SMBT, hindered disulfate linker);

succinimidyl 6-[3-(2-pyridyldithio) propionamido]hexanoate (LC-SPDP); sulfosuccinimidyl 4-

(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC); succinimidyl 3-(2-

pyridyldithio)butyrate (SPDB; hindered disulfide bond linker); sulfosuccinimidyl 2-(7-azido-4-

methylcoumarin-3-acetamide) ethyl-1,3'-dithiopropionate (SAED); sulfo-succinimidyl 7-azido-

10   4-methylcoumarin-3-acetate (SAMCA); sulfosuccinimidyl 6-[alpha-methyl-alpha-(2-

pyridyldithio)toluamido]hexanoate (sulfo-LC-SMPT); 1,4-di-[3'-(2'-

pyridyldithio)propionamido]butane (DPDPB); 4-succinimidyloxycarbonyl-α-methyl-α-(2-

pyridylthio)toluene (SMPT, hindered disulfate linker); sulfosuccinimidyl6[α-methyl-α-(2-

pyridyldithio)toluamido]hexanoate (sulfo-LC-SMPT); *m*-maleimidobenzoyl-N-

15   hydroxysuccinimide ester (MBS); m-maleimidobenzoyl-N-hydroxysulfosuccinimide ester

(sulfo-MBS); N-succinimidyl(4-iodoacetyl)aminobenzoate (SIAB; thioether linker);

sulfosuccinimidyl(4-iodoacetyl)amino benzoate (sulfo-SIAB); succinimidyl4(*p*-

maleimidophenyl)butyrate (SMPB); sulfosuccinimidyl4-(*p*-maleimidophenyl)butyrate (sulfo-

SMPB); azidobenzoyl hydrazide (ABH).

20   Other heterobifunctional cleavable cross-linkers include, N-succinimidyl (4-

iodoacetyl)-aminobenzoate; sulfosuccinimydil (4-iodoacetyl)-aminobenzoate; 4-succinimidyl-

oxycarbonyl-a-(2-pyridyldithio)toluene; sulfosuccinimidyl-6- [a-methyl-a-(pyridyldithiol)-

toluamido] hexanoate; N-succinimidyl-3-(-2-pyridyldithio) - proprionate; succinimidyl 6[3(-(-

2-pyridyldithio)-proprionamido] hexanoate; sulfosuccinimidyl 6[3(-(-2-pyridyldithio)-

25   propionamido] hexanoate; 3-(2-pyridyldithio)-propionyl hydrazide, Ellman's reagent,

dichlorotriazinic acid, S-(2-thiopyridyl)-L-cysteine. Further exemplary bifunctional linking

compounds are disclosed in U.S. Patent Nos. 5,349,066. 5,618,528, 4,569,789, 4,952,394, and

5,137,877.

2.   Exemplary Linkers

30   Any linker known to those of skill in the art for preparation of conjugates may be used

herein. These linkers are typically used in the preparation of chemical conjugates; peptide

linkers may be incorporated into fusion proteins.

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Linkers can be any moiety suitable to associate the mutant analyte binding enzyme and the facilitating agent. Such linkers and linkages include, but are not limited to, peptidic linkages, amino acid and peptide linkages, typically containing between one and about 60 amino acids, more generally between about 10 and 30 amino acids,chemical linkers, such as

5   heterobifunctional cleavable cross-linkers, including but are not limited to, N-succinimidyl (4-iodoacetyl)-aminobenzoate, sulfosuccinimydil (4-iodoacetyl)-aminobenzoate, 4-succinimidyl-oxycarbonyl-a- (2-pyridyldithio)toluene, sulfosuccinimidyl-6- [a-meth ¹-ª-ʔyridyldithiol)-toluamido] hexanoate, N-succinimidyl-3-(-2-pyridyldithio) - proprionate, succinimidyl 6[3(-(-2-pyridyldithio)-proprionamido] hexanoate, sulfosuccinimidyl 6[3(-(-2-pyridyldithio)-

10   propionamido] hexanoate, 3-(2-pyridyldithio)-propionyl hydrazide, Ellman's reagent, dichlorotriazinic acid, and 3-(2-thiopyridyl)-L-cysteine. Other linkers include, but are not limited to peptides and other moieties that reduce stearic hindrance between the mutant analyte binding enzyme and the facilitating agent, intracellular enzyme substrates, linkers that increase the flexibility of the conjugate, linkers that increase the solubility of the conjugate, linkers that

15   increase the serum stability of the conjugate, photocleavable linkers and acid cleavable linkers.

Other exemplary linkers and linkages that are suitable for chemically linked conjugates include, but are not limited to, disulfide bonds, thioether bonds, hindered disulfide bonds, and covalent bonds between free reactive groups, such as amine and thiol groups. These bonds are produced using heterobifunctional reagents to produce reactive thiol groups on one or both of

20   the polypeptides and then reacting the thiol groups on one polypeptide with reactive thiol groups or amine groups to which reactive maleimido groups or thiol groups can be attached on the other. Other linkers include, acid cleavable linkers, such as bismaleimideothoxy propane, acid labile-transferrin conjugates and adipic acid diihydrazide, that would be cleaved in more acidic intracellular compartments; cross linkers that are cleaved upon exposure to UV or visible

25   light and linkers, such as the various domains, such as C_H1, C_H2, and C_H3, from the constant region of human IgG_1 (see, Batra, et al. (1993) Molecular Immunol. 30:379-386). In some embodiments, several linkers may be included in order to take advantage of desired properties of each linker.

Chemical linkers and peptide linkers may be inserted by covalently coupling the linker

30   to the mutant analyte binding enzyme and the facilitating agent. The heterobifunctional agents, described below, may be used to effect such covalent coupling. Peptide linkers may also be linked by expressing DNA encoding the linker and TA, linker and targeted agent, or linker, targeted agent and TA as a fusion protein. Flexible linkers and linkers that increase solubility

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of the conjugates are contemplated for use, either alone or with other linkers are also contemplated herein.

a. Acid cleavable, photocleavable and heat sensitive linkers

5   Acid cleavable linkers, photocleavable and heat sensitive linkers may also be used, particularly where it may be necessary to cleave the targeted agent to permit it to be more readily accessible to reaction. Acid cleavable linkers include, but are not limited to, bismaleimideothoxy propane; and adipic acid dihydrazide linkers (see, *e.g.*, Fattom, *et al.* (1992) *Infection & Immun. 60*:584-589) and acid labile transferrin conjugates that contain a

10   sufficient portion of transferrin to permit entry into the intracellular transferrin cycling pathway (see, *e.g.*, Welhöner, *et al.* (1991) *J. Biol. Chem. 266*:4309-4314).

Photocleavable linkers are linkers that are cleaved upon exposure to light (see, *e.g.*, Goldmacher, *et al.* (1992) *Bioconj. Chem. 3*:104-107, which linkers are herein incorporated by reference), thereby releasing the targeted agent upon exposure to light. Photocleavable linkers

15   that are cleaved upon exposure to light are known (see, *e.g.*, Hazum, *et al.* (1981) in *Pept., Proc. Eur. Pept. Symp.*, 16th, Brunfeldt, K (Ed), pp. 105-110, which describes the use of a nitrobenzyl group as a photocleavable protective group for cysteine; Yen, *et al.* (1989) *Makromol. Chem 190*:69-82, which describes water soluble photocleavable copolymers, including hydroxypropylmethacrylamide copolymer, glycine copolymer, fluorescein copolymer

20   and methylrhodamine copolymer; Goldmacher, *et al.* (1992) *Bioconj. Chem. 3*:104-107, which describes a cross-linker and reagent that undergoes photolytic degradation upon exposure to   )
near UV light (350 nm); and Senter, *et al.* (1985) *Photochem. Photobiol 42*:231-237, which describes nitrobenzyloxycarbonyl chloride cross linking reagents that produce photocleavable linkages), thereby releasing the targeted agent upon exposure to light. Such linkers would have

25   particular use in treating dermatological or ophthalmic conditions that can be exposed to light using fiber optics. After administration of the conjugate, the eye or skin or other body part can be exposed to light, resulting in release of the targeted moiety from the conjugate. Such photocleavable linkers are useful in connection with diagnostic protocols in which it is desirable to remove the targeting agent to permit rapid clearance from the body of the animal.

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b. Other linkers for chemical conjugation

Other linkers, include trityl linkers, particularly, derivatized trityl groups to generate a genus of conjugates that provide for release of therapeutic agents at various degrees of acidity or alkalinity. The flexibility thus afforded by the ability to preselect the pH range at which the

5   therapeutic agent will be released allows selection of a linker based on the known physiological differences between tissues in need of delivery of a therapeutic agent (see, *e.g.*, U.S. Patent No. 5,612,474). For example, the acidity of tumor tissues appears to be lower than that of normal tissues.

c. Peptide linkers

10   The linker moieties can be peptides. Petide linkers can be employed in fusion proteins and also in chemically linked conjugates. The peptide typically a has from about 2 to about 60 amino acid residues, for example from about 5 to about 40, or from about 10 to about 30 amino acid residues. The length selected will depend upon factors, such as the use for which the linker is included.

15   The proteinaceous ligand binds with specificity to a receptor(s) on one or more of the target cell(s) and is taken up by the target cell(s). In order to facilitate passage of the chimeric ligand-toxin into the target cell, it is presently preferred that the size of the chimeric ligand-toxin be no larger than can be taken up by the target cell of interest. Generally, the size of the chimeric ligand-toxin will depend upon its composition. In the case where the chimeric ligand

20   toxin contains a chemical linker and a chemical toxin (i.e., rather than proteinaceous one), the size of the ligand toxin is generally smaller than when the chimeric ligand-toxin is a fusion protein. Peptidic linkers can conveniently be encoded by nucleic acid and incorporated in fusion proteins upon expression in a host cell, such as *E. coli*.

Peptide linkers are advantageous when the facilitating agent is proteinaceous. For

25   example, the linker moiety can be a flexible spacer amino acid sequence, such as those known in single-chain antibody research. Examples of such known linker moieties include, but are not limited to, peptides, such as $(Gly_mSer)_n$ and $(Ser_mGly)_n$, in which n is 1 to 6, preferably 1 to 4, more preferably 2 to 4, and m is 1 to 6, preferably 1 to 4, more preferably 2 to 4, enzyme cleavable linkers and others.

30   Additional linking moieties are described, for example, in Huston, *et al.*, *Proc. Natl. Acad. Sci. U.S.A. 85*:5879-5883, 1988; Whitlow, M., *et al.*, *Protein Engineering 6*:989-995, 1993; Newton, *et al.*, *Biochemistry 35*:545-553, 1996; A. J. Cumber, *et al.*, *Bioconj. Chem.*

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3:397-401, 1992; Ladurner, et al., J. Mol. Biol. 273:330-337, 1997; and U.S. Patent. No. 4,894,443. In some embodiments, several linkers may be included in order to take advantage of desired properties of each linker.

N.   PREPARATION OF CONJUGATES

5   Conjugates with linked targeted agents can be prepared either by chemical conjugation, recombinant DNA technology, or combinations of recombinant expression and chemical conjugation. The mutant analyte binding enzyme and the facilitating agent may be linked in any orientation and more than one targeting agent and/or targeted agent may be present in a conjugate.

10   **1.**   **Selection of facilitating agents**

Any agent that facilitates detection, immobilization, or purification of the conjugate is contemplated for use herein. For chemical conjugates any moiety that has such properties is contemplated; for fusion proteins, the facilitating agent is a protein, peptide or fragment thereof that is sufficient to effect the facilitating activity.

15   **a.**   **Protein binding moieties**

The conjugate contains a protein binding moiety, particularly a protein binding protein, peptide or effective fragment thereof. Its specific binding partner can be proteins or peptides generally, a set of proteins or peptides or mixtures thereof, or a particular protein or peptide. Any protein-protein interaction pair known to those of skill in the art is contemplated. For

20 example, the protein-protein interaction pair can be enzyme/protein or peptide substrate, antibody/protein or peptide antigen, receptor/protein or peptide ligand, etc. Any protein-protein interaction pair can be designed, screened or selected according to the methods known in the art (See generally, *Current Protocols in Molecular Biology* (1998) § 20, John Wiley & Sons, Inc.). Examples of such methods for identifying protein-protein interactions include the interaction

25 trap/two-hybrid system and the phage-based expression cloning.

1)   Interaction trap/two-hybrid system

Interacting proteins can be identified by a selection or screen in which proteins that specifically interact with a target protein of interest are isolated from a library. One particular approach to detect interacting proteins is the two-hybrid system or interaction trap (See

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generally, *Current Protocols in Molecular Biology* (1998) § 20.1.-20.2., John Wiley & Sons, Inc.), which uses yeast as a "test tube" and transcriptional activation of a reporter system to identify associating proteins.

In the two-hybrid system, a yeast vector such as the plasmid pEG202 or a related vector

5   can be used to express the probe or "bait" protein as a fusion to the heterologous DNA-binding protein LexA. Many proteins, including transcription factors, kinases, and phosphatases, can be used as bait proteins. The major requirements for the bait protein are that it should not be actively excluded from the yeast nucleus, and it should not possess an intrinsic ability to strongly activate transcription. The plasmid expressing the LexA-fused bait protein can be

10   used to transform yeast possessing a dual reporter system responsive to transcriptional activation through the *LexA* operator.

In one such example, the yeast strain EGY48 containing the reporter plasmid pSH18-34 can be used. In this case, binding sites for LexA are located upstream of two reporter genes. In the EGY48 strain, the upstream activating sequences of the chromosomal *LEU2* gene, which is

15   required in the biosynthetic pathway for leucine (Leu), are replaced with *Lex*A operators (DNA binding sites). PSH18-34 contains a *Lex*A operator-*lacZ* fusion gene. These two reporters allow selection for transcriptional activation by permitting selection for viability when cells are plated on medium lacking Leu, and discrimination based on color when the yeast is grown on medium containing Xgal.

20   The EGY48/PSH18-34 transformed with a bait is first characterized for its ability to express protein, growth on medium lacking Leu, and for the level of transcriptional activation of *lacZ*. A number of alternative strains, plasmids, and strategies can be employed if a bait proves to have an unacceptably high level of background transcriptional activation.

In an interactor hunt, the stain EGY48/PSH18-34 containing the bait expression plasmid

25   is transformed, preferably along with carrier DNA, with a conditionally expressed library made in a suitable vector such as the vector pJG4-5. This library uses the inducible yeast GAL1 promoter to express proteins as fusions to an acidic domain ("acid blob") that functions as a portable transcriptional activation motif (act) and to other useful moieties. Expression of library-encoded proteins is induced by plating transformants on medium containing galactose

30   (Gal), so yeast cells containing library proteins that do not interact specifically with the bait protein will fail to grow in the absence of Leu. Yeast cells containing library proteins that interact with the bait protein will form colonies within 2 to 5 days, and the colonies will turn blue when the cells are streaked on medium containing Xgal. The DNA from interaction trap

positive colonies can be analyzed by polymerase chain reaction (PCR) to streamline screening

and detect redundant clones in cases where many positives are obtained in screening. The

plasmids can be isolated and characterized by a series of tests to confirm specificity of the

interaction with the initial bait protein.

5   An alternative way of conducting an interactor hunt is to mate a strain that expresses the

bait protein with a strain that has been pretransformed with the library DNA, and screen the

resulting diploid cells for interactors (Bendixen, et al., Nucl. Acids. Res., 22:1778-1779 (1994);

and Finley and Brent, Proc. Natl. Sci. U.S.A., 91:12980-12984 (1994)). This "interaction

mating" approach can be used for any interactor hunt, and is particularly useful in three special

10   cases. The first case is when more than one bait will be used to screen a single library.

Interaction mating allows several interactor hunts with different baits to be conducted using a

single high-efficiency yeast transformation with library DNA. This can be a considerable

savings, since the library transformation is one of the most challenging tasks in an interactor

hunt. The second case is when a constitutively expressed bait interferes with yeast viability.

15   For such baits, performing a hunt by interaction mating avoids the difficulty associated with

achieving a high-efficiency library transformation of a strain expressing a toxic bait.

Moreover, the actual selection for interactors will be conducted in diploid yeast, which are

more vigorous than haploid yeast and can better tolerate expression of toxic proteins. The third

case is when a bait cannot be used in a traditional interactor hunt using haploid yeast stains

20   because it activates transcription of even the least sensitive reporters. In diploids the reporters

are less sensitive to transcription activation than they are in haploids. Thus, the interaction

mating hunt provides an additional method to reduce background from transactivating baits.

          The interaction trap/two-hybrid system and the identified protein-protein interaction

pairs have been successfully used (see, e.g., Bartel, et al., Using the two-hybrid system to

25   detect protein-protein interactions, In Cellular Interactions in Development: A Practical

Approach, (D.A. Hartley, ed.) pp. 153-179, Oxford University Press, Oxford (1993); Bartel, et

al., A protein linkage map of Escherichia coli bacteriophage T7, Nature Genet., 12:72-77

(1996); Bendixen, et al., A yeast mating-selection scheme for detection of protein-protein

interactions, Nucl. Acids. Res., 22:1778-1779 (1994); Breeden and Nasmyth, Regulation of the

30   yeast HO gene., Cold spring Harbor Symp. Quant. Biol, 50:643-650 (1985); Brent and Ptashne,

A bacterial repressor protein or a yeast transcriptional terminator can block upstream activation

of a yeast gene, Nature, 312:612-615 (1984); Brent, et al., A eukaryotic transcriptional

activator bearing the DNA specificity of a prokaryotic repressor, Cell, 43:729-736 (1985);

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Chien, *et al.*, The two-hybrid system: A method to identify and clone genes for proteins that interact with a protein of interest, *Proc. Natl. Acad. Sci. U.S.A., 88*:9578-9582 (1991); Chiu, *et al.*, RAPT1, a mammalian homolog of yeast Tor, interacts with the FKBP12/rapamycin complex, *Proc. Nat. Acad. Sci., U.S.A., 91*:12574-12578 (1994); Colas, *et al.*, Genetic selection

5   of peptide aptamers that recognize and inhibit cyclin-dependent kinase 2., *Nature, 380*:548-550 (1996); Durfee, *et al.*, The retinoblastoma protein associates with the protein phosphatase type 1 catalytic subunit, *Genes & Dev., 7*:555-569 (1993); Estojak, *et al.*, Correlation of two-hybrid affinity data with in vitro measurements, *Mol. Cell. Biol., 15*:5820-5829 (1995); Fearon, *et al.*, Karyoplasmic interaction selection strategy: A general strategy to detect protein-protein

10   interaction in mammalian cells, *Proc. Nat., Acad. Sci. U.S.A., 89*:7958-7962 (1992); Fields and Song, A novel genetic system to detect protein-protein interaction, *Nature, 340*:245-246 (1989); Finley and Brent, Interaction mating revels binary and ternary connections between *Drosophila* cell cycle regulators, *Proc. Natl. Sci. U.S.A., 91*:12980-12984 (1994); Gietz, *et al.*, Improved method for high-efficiency transformation of intact yeast cells, *Nucl. Acids. Res.*,

15   *20*:1425 (1992); Golemis and Brent, Fused protein domains inhibit DNA biding by LexA, *Mol. Cell Biol., 12*:3006-3014 (1992); Gyuris, *et al.*, Cdi1, a human G1 and S-phase protein phosphatase that associates with Cdk1, *Cell, 75*:791-803 (1993); Kaiser, *et al.*, A., Methods in Yeast Genetics, a Cold Spring Harbor Laboratory Course Manual, pp. 135-136. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1994); Kolonin and Finley, Jr., Targeting

20   cyclin-dependent kinases in *Drosophila* with peptide aptamers, *Proc. Natl. Acad. Sci. U.S.A., In press* (1998); Licitra and Liu, A three-hybrid system for detecting small ligand-protein receptor interactions, *Proc. Nat. Acad. Sci. U.S.A., 93*:12817-12821 (1996); Ma and Ptashne, A new class of yeast transcriptional activators, *Cell, 51*:113-119 (1987); Ma and Ptashne, Converting an eukaryotic transcriptional inhibitor into an activator, *Cell, 55*:443-446 (1988); Osborne, *et*

25   *al.*, The yeast tribrid system: Genetic detection of transphosphorylated ITAM-SH2 interactions, *Bio/Technology, 13*:1474-1478 (1995); Ruden, *et al.*, Generating yeast transcriptional activators containing no yeast protein sequences, *Nature, 350*:426-430 (1991); Samson, *et al.*, Gene activation and DNA binding by *Drosophila* Ubx and abd-A proteins, *Cell, 57*:1045-1052 (1989); Stagljar, *et al.*, Use of the two-hybrid system and random sonicated DNA to identify

30   the interaction domain of a protein, *BioTechniques, 21*:430-432 (1996); Vasavada, *et al.*, A contingent replication assay for the detection of protein-protein interactions in animal cells, *Proc. Nat. Acad. Sci. U.S.A., 88*:10686-10690 (1991); Vojtex, *et al.*, Mammalian Ras interacts directly with the serine/threonine kinase Raf, *Cell, 74*:205-214 (1993); Watson, *et al.*, Vectors

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encoding alternative antibiotic resistance for use in the yeast two-hybrid system, *BioTechniques, 21*:255-259 (1996); West, *et al., Saccharomyces cerevisiae GAL10* divergent promoter region: Location and function of the upstream activator sequence UASG, *Mol. Cell Biol., 4*:2467-2478 (1984); and Yang, *et al.*, Protein-peptide interactions analyzed with the
5   yeast two-hybrid system, *Nucl. Acids Res., 23*:1152-1156 (1995)) and can be used in the present system.

2)   Phage-based expression cloning

Interaction cloning (also known as expression cloning) is a technique to identify and clone genes that encode proteins that interact with a protein of interest, or "bait" protein.
10   Phage-based interaction cloning requires a gene encoding the bait protein and an appropriate expression library constructed in a bacteriophage expression vector, such as λgt11 (See generally, *Current Protocols in Molecular Biology* (1998) § 20.3, John Wiley & Sons, Inc.). The gene encoding the bait protein is used to produce recombinant fusion protein in *E. coli*. The cDNA is radioactively labeled with ^{32}P. A recognition site for a protein kinase such as the
15   cyclic adenosine 3',5'-phosphate (cAMP)—dependent protein kinase (Protein kinase A; PKA) is introduced into the recombinant fusion protein to allow its enzymatic phosphorylation by the kinase and [λ-^{32}P]ATP.

In one example, the procedure involves a fusion protein containing bait protein and glutathione-S-transferase (GST) with a PKA site at the junction between them. The labeled
20   protein is subsequently used as a probe to screen a λ bacteriophage-derived cDNA expression library, which expresses β-galactosidase fusion proteins that contain in-frame gene fusions. The phages lyse cells, form plaques, and release fusion proteins that are adsorbed onto nitrocellulose membrane filters. The filters are blocked with excess nonspecific protein to eliminate nonspecific binding and probed with the radiolabeled bait protein. This procedure
25   leads directly to the isolation of genes encoding the interacting protein, by passing the need for purification and microsequencing or for antibody production.

The phage-based interaction cloning system and the identified protein-protein interaction pairs have been successfully employed (Blanar, *et al.*, Interaction cloning: Identification of a helix-loop-helix zipper protein that interacts with c-Fos, *Science, 256*:1014-
30   1018 (1992); Carr and Scott, Blotting and band-shifting: Techniques for studying protein-protein interactions, *Trends Biochem. Sci., 17*:246-249 (1992); Chapline, *et al.*, Interaction cloning of protein kinase C substrates, *J. Biol. Chem., 268*:6858-6861 (1993); Hoeffler, *et al.*,

Identification of multiple nuclear factors that interact with cyclic AMP response element-binding protein and activation transcription factor-2 by protein interactions, *Mol. Endocrinol.*, *5*:256-266 (1991); Kaelin, *et al.*, Expression cloning of a cDNA encoding a retinoblastoma-binding protein with E2F-like properties, *Cell, 70*:351-364 (1992); Lester, *et al.*, Cloning and

5   characterization of a novel A-kinase anchoring protein: AKAP220, association with testicular peroxisomes, *J. Biol. Chem., 271*:9460-9465 (1996); Lowenstein, *et al.*, The SH2 and SH2 domain-containing protein GRB2 links receptor tyrosine kinase to ras si⌐nali⌐ʒ, *Cell, 70*:431-442 (1992); Margolis, *et al.*, High-efficiency expression/cloning of epidermal growth factor-receptor-binding proteins with *src* homology 2 domains, *Proc. Natl. Acad. Sci. U.S.A.*,

10 *89*:8894-8898 (1992); Skolnik, *et al.*, Cloning of P13 kinase-associated p85 utilizing a novel method of expression/cloning of target proteins for receptor tyrosine kinases, *Cell, 65*:83-90 (1991); and Stone, *et al.*, Interaction of a protein phosphatase with an *Arabidopsis* serine-threonine receptor kinase, *Science, 266*:793-795 (1994)) and can be used in the present system.

3)   Detection of protein-protein interactions

15   Surface plasmon resonance (SPR) can be used to verify the protein-protein interactions identified by other systems such as the interaction trap/two-hybrid system and the phage-based expression cloning systems (See generally, *Current Protocols in Molecular Biology* (1998) § 20.4, John Wiley & Sons, Inc.). This is an *in vitro* technique based on an optical phenomenon, called SPR, that can simultaneously detect interactions between unmodified proteins and

20 directly measure kinetic parameters of the interaction.

        SPR devices are commercially available. The BIAcore instrument (BIAcore) is presently preferred herein. This instrument contains sensing optics, an automated sample delivery system, and a computer for instrument control, data collection, and data processing. Experiments are performed on disposable chips. In practice, a ligand protein is immobilized on

25 the chip while buffer continuously flows over the surface. The sensing apparatus monitors changes in the angle of minimum reflectance from the interface that result when a target protein associates with the ligand protein. Molecular interactions can be directly visualized (on the computer monitor) in real time as the optical response is plotted against time. This response is measured in resonance units (RUs, where 1000 RUs = 1 ng protein/mm^2).

30   The SPR system has been successfully used (see, *e.g.*, BioSupplyNet Source Book, BioSupplyNet, Plainview, N.Y., and Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1999); Feng, *et al.*, Functional binding between Gβ and the LIM domain of Ste5

is required to activate the MEKK Ste11, *Cur. Biol.*, 8:267-278 (1998); Field, *et al.*, Purification of RAS-responsive adenylyl cyclase complex from *Sacchariomyces cerevisiae* by use of an epitope addition method, *Mol. Cell. Biol.*, 8:2159-2165 (1988); Phizicky and Fields, Protein-protein interactions: Methods for detection and analysis, *Microbiol. Rev.*, 59:94-123 (1995);

5   Tyers, *et al.*, Comparison of the *Saccharomyces cerevisiae* G1 cyclins: Cln3 may be an upstream activator of Cln1, Cln2, and other cyclins, *EMBO J.*, 11:1773-1784 (1993)) and the identified protein-protein interaction pairs can be used in the present system.

b.   Epitope tags

The facilitating agent can be any moiety, particularly a protein, peptide or effective

10   fragment thereof that is specifically recognized by an antibody. In these embodiments, the conjugate contains an epitope tag that is specifically recognized by a set of antibodies or by a particular antibody. Any epitope/antibody pair can be used in the present system (See generally, *Current Protocols in Molecular Biology* (1998) 10.15, John Wiley & Sons, Inc.). The following Table 4 provides exemplary epitope tags and illustrates certain properties of

15   several commonly used epitope tag systems.

Table 4.   Exemplary epitope tag systems

Epitope	Peptide	SEQ ID	Antibody	Reference
FLAG	AspTyrLysAspAspAspLys	63	4E11	Prickett[1]
HA	TyrProTyrAspValPRoAspTyrAla	64	12Ca5	Xie[2]
HA1	CysGlnAspLeuProGlyAsnAspAsnSerThr	65	mouse MAb	Nagelkerken[3]
c-Myc	GluGlnLysLeuIleSerGluGluAspLeu	66	9E10	Xie[2]
6-His	HisHisHisHisHisHis	67	BAbCO*	
AU1	AspThrTyrArgTyrIle	68	BAbCO	
EE	GluTyrMetProMetGlu	69	anti-EE	Tolbert[4]
T7	AlaSerMetThrGlyGlyGlnGlnMetGlyArg	70	Invitrogen	Chen[5] Tseng[6]
4A6	SerPheProGlnPheLysProGlnGluIle	71	4A6	Rudiger[7]
ε	LysGlyPheSerTyrPheGlyGluAspLeuMetPro	72	anti-PKCε	Olah[8]
B	GlnTyrProAlaLeuThr	73	D11, F10	Wang[9]
gE	GlnArgGlnTyrGlyAspValPheLysGlyAsp	74	3B3	Grose[10]
Ty1	GluValHisThrAsnGlnAspProLeuAsp	75	BB2, TYG5	Bastin[11]

1.   Prickett, *et al.*, *BioTechniques*, 7(6):580-584 (1989)
2.   Xie, *et al.*, *Endocrinology*, 139(11):4563-4567 (1998)
3.   Nagelkerke, *et al.*, *Electrophoresis*, 18:2694-2698 (1997)
20 4.   Tolbert and Lameh, *J. Neurochem.*, 70:113-119 (1998)
5.   Chen and Katz, *BioTechniques*, 25(1):22-24 (1998)
6.   Tseng and Verma, *Gene*, 169:287-288 (1996)

7.　　Rudiger, *et al.*, *BioTechniques*, 23(1):96-97 (1997)

8.　　Olah, *et al.*, *Biochem.*, 221:94-102 (1994)

9.　　Wang, *et al.*, *Gene*, 169(1):53-58 (1996)

10.　　Grose, U.S. Patent No. 5,710,248

11.　　Bastin, *et al.*, *Mol. Biochem. Parasitology*, 77:235-239 (1996)

Invitrogen, Sigma, Santa Cruz Biotech

For example, in one embodiment, the selected epitope tag is the 6-His tag. Vectors for constructing a fusion protein containing the 6-His tag and reagents for isolating or purifying such fusion proteins are commercially available. For example, the Poly-His gene fusion vector from Invitrogen, Inc. (Carlsbad, CA) includes the following features: 1) high-level regulated transcription for the *trc* promotor; 2) enhanced translation efficiency of eukaryotic genes in *E.coli*; 3) the *LacO* operator and the *LacI*^{q} repressor gene for transcriptional regulation in any *E. coli* system; N-terminal Xpress epitope for easy detection with an Anti-Xpress antibody; and 4) enterokinase cleaving site for removal of the fusion tag. The fusion protein can be purified by nickel-chelating agarose resin, and the purified fusion protein can be coated onto a microtiter plate pre-coated with nickel (*e.g.*, Reacti-Binding meta chelate polystyrene plates, Pierce) for diagnostic usage.

In addition, the fusion protein containing the 6-His tag can be isolated or purified using the His MicroSpin Purification Module or HisTrap Kit from Amersham Pharmacia Biotech, Inc. The His MicroSpin Purification Module provides fifty MicroSpin columns prepacked with nickel-charged Chelating Sepharose Fast Flow. The module enables the simple and rapid screening of large numbers of small-scale bacterial lysates for the analysis of putative clones and optimization of expression and purification conditions. Each column contains 50 μl bed volume, enough to purify > 100 μg his-tagged fusion protein, from up to 400 μl of His-tagged fusion protein sample, *e.g.*, crude lysate and purification intermediates. The HisTrap Kit is designed for rapid, mild affinity purification of histidine-tagged fusion proteins in a single step. The high dynamic capacity of HiTrap Chelating enables milligrams of protein to be purified in less than 15 minutes at flow rates of up to 240 column volumes per hour. The high capacity is maintained after repeated use ensuring cost-effective, reproducible purifications. The Kit includes three HiTrap Chelating columns and buffer concentrates to perform F10-12 purifications with a syringe. The anti-His antibody from Amersham Pharmacia Biotech, Inc. is an IgG$_2$ subclass of monoclonal antibody directed against 6 Histidine residues. The antibody is unconjugated to offer the flexibility of detection with a secondary antibody conjugated with

either horseradish peroxidase or alkaline phosphatase. The antibody provides high sensitivity
with low background.

Further examples of epitope tagging can be found in Kolodziej and Young, Epitope
tagging and protein surveillance, *Methods Enzymol.*, 194:508-519 (1991). Methods for

5   preparing and using other such tags and other such tags similarly can be used in the methods
and products provided herein.

c.   IgG binding proteins

In other embodiments, the conjugate contains an IgG binding protein, which, for
example provides a means for selective binding of the conjugate. Any IgG binding protein/IgG

10 pair can be used in the present system. Protein A and Protein G are suitable facilitating. Any
Protein A or Protein G can be used in the present system.

For example, the following nucleotide sequences can be used for amplifying and
constructing Protein A or Protein G fusion proteins: E04365 (Primer for amplifying IgG
binding domain AB of protein A); E04364 (Primer for amplifying IgG binding domain AB of

15 protein A); E01756 (DNA sequence encoding subunit which can bind IgG of protein A like
substance); M74187 (Cloning vector pKP497 (cloning, screening, fusion vector) encoding an
IgG-binding fusion protein from protein A analogue (ZZ) and beta-Gal'(lacZ) genes). In
addition, several Protein A gene fusion vectors such as pEZZ 18 and pRIT2T are commercially
available (Amersham Pharmacia Biotech, Inc.).

20   ### 1)   pEZZ 18 Protein A gene fusion vector

pEZZ 18 Protein A gene fusion vector can be used for rapid expression of secreted
fusion proteins and their one-step purification using IgG Sepharose 6FF. The phagemid pEZZ
18 contains the proteins A signal sequence and two synthetic "Z" domains based on the "B"
IgG binding domain of Protein A (Löwenadler, *et al.*, *Gene*, 58:87 (1987); and Nilsson, *et al.*,

25 *Prot. Engineering*, 1:107 (1987)). Proteins are expressed as fusions with the "ZZ" peptide and
secreted into the aqueous culture medium under the direction of the protein A signal sequence.
They are easily purified using IgG Sepharose 6FF to which the "ZZ" domain binds tightly.
Because of its unique folding properties, the 14 kDa "ZZ" peptide has little effect on folding of
the fusion partner into a native conformation.

Expression

Expression is controlled by the *lac*UV5 and protein A promotors and is not inducible. Elements of the protein A gene provide the ATG and ribosome-binding sites. Stop codons must be provided by the insert.

Sequencing

The M13 Universal Sequencing Primer is used for double-stranded and single-stranded sequencing. A protocol for production of single-stranded DNA is provided with the vector.

Cloning

Inserts containing a stop codon will yield white colonies when grown on media containing X-gal.

Host(s)

E. coli strains carrying a *lac* deletion but capable of α-complementation of *lacZ'*.

Selectable marker(s)

Plasmid confers resistance to ampicillin.

Amplification

Amplification, though not necessarily required can be included.

2)   pRIT2T Protein A gene fusion vector

The pRIT2T Protein A gene fusion vector (available from Pharmacia) can be used for high-level expression of intracellular fusion proteins. pRIT2T, a derivative of pRIT2 (Nilsson, *et al.*, *EMBO J.*, 4:1075 (1985)), contains the IgG-binding domains of *staphylococcal* protein A which permits rapid affinity purification of fusion proteins on IgG Sepharose 6 FF. Thermo-inducible expression of the fusion protein is achieved in a suitable *E. coli* host strain which carries the temperature-sensitive repressor *cI*857 (N4830-1) (Zabeau and Stanley, *EMBO J.*, 1:1217 (1982)).

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Induction

The λP$_R$ promoter is induced by shifting the growth temperature from 30°C to 42°C for 90 minutes.

Expression

5   Genes inserted into the MCS are expressed from the λ right promoter (P$_R$) as fusions with the IgG-binding domains of *staphylococcal* protein A. A portion of the λ *cro* gene, fused to the IgG-binding domain, supplies the ATG start codon. Since no signal sequence is provided, the protein remains intracellular. Protein A gene transcription and translation termination signals are provided. Fusion protein can be purified on IgG

10   Sepharose 6FF (17-0969-01). The protein A carrier protein is ~30 kDa.

Host(s)

E. coli N4830-1/N99cI$^+$. Supplied with *E. coli* N4830-1 which contains the temperature-sensitive *cI857* repressor.

Selectable marker(s)

15   Plasmid confers resistance to ampicillin.

3)   The IgG Sepharose 6 fast flow system

The Protein A and Protein G fusion protein can be isolated or purified by affinity binding with IgG, such as the IgG Sepharose 6 Fast Flow System (Amersham Pharmacia Biotech, Inc.). The IgG Sepharose 6 Fast Flow System includes IgG coupled to the highly

20   cross-linked 6% agarose matrix Sepharose 6 Fast Flow, and is designed for the rapid purification of Protein A and Protein A fusion conjugates. The system binds at least 2 mg Protein A/ml drained gel with flow possible rates of 300 cm/hr at 1 bar (14.5 psi, 0.1 MPa) in an XK 50/30 column (Lundström, *et al.*, *Biotechnology and Bioengineering*, 36:1056 (1990)).

d.   β-galatosidase fusion proteins

25   The pMC1871 fusion vector (commercially available from Pharmacia, see, also Shapira, *et al.*, *Gene* 25:71 (1983); Casadaban, *et al.*, *Methods Enzymol. 100*:293 (1983)) for production of enzymatically active β-galactosidase hybrid proteins for gene expression or functional studies. Vector pMC1871 is derived from pBR322 and contains a promoterless lacZ

gene, which also lacks a ribosome-binding site and the first eight non-essential N-terminal amino acid codons. Its unique Sma I site allows fusions to the N-terminal part of the β-galactosidase gene. Insertion of a gene into the *E. coli* lacZ gene results in the production of a hybrid protein, whose presence can be readily detected by following its β galactosidase activity (Miller, J.H., in Experiments in Molecular Gener. (Cold Spring Harbor, N.Y.) (1972); Nielsen, *et al., Proc. Natl. Acad. Sci. U.S.A.*, *80*:5198 (1983)). Hybrid proteins can then be easily purified by affinity chromatography (Germino, *et al., Proc. Natl. Acad. Sci. U.S.A., 81*: 4692 (1984)). Multiple cloning sites flanking the lacZ gene permit its excision as a BamH I, Sal I, Pst I or EcoR I gene cassette. If lacZ is excised as an EcoRI cassette, a portion of its 3'-end will be deleted. The resulting β-glactosidase protein (α-donor) will be functional if the C-terminus of the β-galactosidase protein (α-acceptor) is available through intercistronic complementation.

Expression

Inserts cloned into the unique Sma I site give fusion proteins with the N-terminal part of β-galactosidase. Insert must contain a promoter, ATG and ribosome-binding site.

Host(s)

E. coli strains carrying a lac deletion.

Selectable marker(s)

Plasmid confers resistance to 15 μg/ml tetracycline.

GenBank Accession Number L08936.

e.   Nucleic acid binding moieties

In another embodiment, the conjugate includes a nucleotide binding protein, peptide or effective fragment thereof as a facilitating agent. The specific binding partner can be nucleotide sequences generally, a set of nucleotide sequences or a particular nucleotide sequence. Any protein-nucleotide interaction pair can be used in the present system. For example, the protein-nucleotide interaction pair can be protein/DNA or protein/RNA pairs, or a combination thereof. Protein-nucleotide interaction pairs can be designed, screened or selected according to the methods known in the art (See generally, *Current Protocols in Molecular Biology* (1998) § 12, John Wiley & Sons, Inc.). Examples of such methods for identifying protein-nucleotide interactions include the gel mobility shift assay, methylation and uracil

interference assay, DNase I footprint analysis, λgt11 expression library screening and rapid
separation of protein-bound DNA from free DNA using nitrocellulose filters.

1)   DNA binding proteins

The conjugate can contain a DNA binding protein and its specific binding partner can
be DNA molecules generally, a set of DNA molecules or a particular sequence of nucleotides.
Any DNA binding protein can be used in the present system. For example, the DNA binding
protein can bind to a single-stranded or double-stranded DNA sequence, or to an A-, B- or Z-
form DNA sequence. The DNA binding sequence can also bind to a DNA sequence that is
involved in replication, transcription, DNA repair, recombination, transposition or DNA
structure maintenance. The DNA binding sequence can further be derived from a DNA
binding enzyme such as a DNA polymerase, a DNA-dependent RNA polymerase, a DNAase, a
DNA ligase, a DNA topoisomerase, a transposase, a DNA kinase, or a restriction enzyme.

Any DNA binding sequence/DNA sequence pair can be designed, screened or selected
according to the methods known in the art including methods described in Section L.2. above.

The following Table 5 illustrates certain properties of several DNA binding
sequence/DNA sequence pair systems.

Table 5.   Examples of DNA binding sequence/DNA sequence binding pairs

DNA binding sequence	DNA binding sequence motif	DNA sequence	Reference (U.S. Patent No.)
NF-AT$_p$ (SEQ ID NO. 76)	T lymphocyte DNA-binding protein	GCCCAAAGAGGAA AATTTGTTTCATAC AG (SEQ ID NO. 77)	5,656,452
Max (SEQ ID NO. 78)	helix-loop-helix zipper protein	CACGTG	5,693,487
Chicken Lung 140 Kd Protein		Z-DNA	5,726,050
EGR1, EGR2, GLI, Wilm's tumor gene, Sp1, Hunchback, Kruppel, ADR1 and BrLA	Zinc finger proteins	GACC, GCAC	5,789,538
LIL-Stat protein	Stat family of transcription factors	TTNCNNAGA, TTCCTGAGA	5,821,053
Egr (SEQ ID NO. 79)	zinc finger protein	CGCCCCCGC	5,866,325

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DNA binding sequence	DNA binding sequence motif	DNA sequence	Reference (U.S. Patent No.)
S1-3 protein (SEQ ID NO. 80)	zinc finger protein	CATRRWWG	5,905,146

2) RNA binding proteins

In another preferred embodiment, the conjugate can contain an RNA binding protein and its specific binding partner can be RNA generally, a set of RNA molecules or a particular

5 sequence of ribonucleotides. Any RNA binding protein can be used in the present system. For example, the RNA binding protein can bind to a single-stranded or double-stranded RNA, or to rRNA, mRNA or tRNA. The RNA binding protein may specifically bind to a RNA that is involved in reverse transcription, transcription, RNA editing, RNA splicing, translation, RNA stabilization, RNA destabilization, or RNA localization. The RNA binding protein can be

10 derived from or be an RNA binding enzyme such as a RNA-dependent DNA polymerase, a RNA-dependent RNA polymerase, a RNase, a RNA ligase, a RNA maturase, or a ribosome.

Other RNA recognition sequence or binding motifs that can be used in the present system include the zinc-finger motif, the Y-box, the KH motif, AUUUA, histone, RNP motif (U1), arginine-rich motif (ARM or PRE), double-stranded RNA binding motifs (IRE) and RGG

15 box (APP) (U.S. Patent Nos. 5,834,184, 5,859,227 and 5,858,675). The RNP motif is a 90-100 amino acid sequence that is present in one or more copies in proteins that bind pre mRNA, mRNA, pre-ribosomal RNA and snRNA. The consensus sequence and the sequences of several exemplary proteins containing the RNP motif are provided in Burd and Dreyfuss, *Science*, 265:615-621 (1994); Swanson, *et al.*, *Trends Biochem. Sci.*, 13:86 (1988); Bandziulis,

20 *et al.*, *Genes Dev.*, 3:431 (1989); and Kenan, *et al.*, *Trends Biochem. Sci.*, 16:214 (1991). The RNP consensus motif contains two short consensus sequences RNP-1 and RNP-2. Some RNP proteins bind specific RNA sequences with high affinities (dissociation constant in the range of 10^{-8}-10^{-11} M). Such proteins often function in RNA processing reactions. Other RNP proteins have less stringent sequence requirements and bind less strongly (dissociation constant about

25 10^{-6}-10^{-7} M) (Burd & Dreyfuss, *EMBO J.*, 13:1197 (1994)).

A second characteristic RNA binding motif found in viral, phage and ribosomal proteins is an arginine-rich motif (ARM) of about 10-20 amino acids. RNA binding proteins having this motif include the HIV Tat and Rev proteins. Rev binds with high affinity disassociation constant (10^{-9} M) to an RNA sequence termed RRE, which is found in all HIV

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mRNAs (Zapp, *et al.*, *Nature*, 342:714 (1989); and Dayton, *et al.*, *Science*, 246:1625 (1989)).
Tat binds to an RNA sequence termed TAR with a dissociation constant of $5X10^{-9}$ M
(Churcher, *et al.*, *J. Mol. Biol.*, 230:90 (1993)). For Tat and Rev proteins, a fragment
containing the arginine-rich motif binds as strongly as the intact protein. In other RNA binding

5 proteins with ARM motifs, residues outside the ARM also contribute to binding.

The double-stranded RNA-binding domain (dsRBD) exclusively binds double-stranded
RNA or RNA-DNA. A dsRBD motif includes a region of approximately 70 amino acids which
includes basic residues and contains a conserved core sequence with a predicted α-helical
structure. The dsRBD motif is found in at least 20 known or putative RNA-binding proteins

10 from different organisms. There are two types of dsRBDs; Type A, which is homologous
along its entire length with the defined consensus sequence, and Type B, which is more highly
conserved at its C terminus than its N terminus. These domains have been functionally
delineated in specific proteins by deletion analysis and RNA binding assays (St Johnston, *et al.*,
Proc. Natl. Acad. Sci., 89:10979-10983 (1992)).

15 Any RNA binding sequence/RNA sequence pair can be designed, screened or selected
according to the methods known in the art including the methods described in Section L.2.
above and the methods, such as those decribed in U.S. Patent Nos. 5,834,184 and 5,859,227,
and in SenGupta, *et al.*, A three-hybrid system to detect RNA-protein interactions in vivo,
Proc. Nat. Acad. Sci. U.S.A., 93:8496-8501 (1996)).

20 For example, U.S. Patent No. 5,834,184 describes a method of screening a plurality of
polypeptides for RNA binding activity. The method includes the steps of: (1) culturing a
library of procaryotic cells that constitute a library, and (2) detecting expression of the reporter
gene in a cell from the library, the expression indicating that the cell comprises a polypeptide
having RNA binding activity. The cells contain at least one vector that contains a first DNA

25 segment that encodes a fusion protein of a prokaryotic anti-terminator protein having anti-
terminator activity linked in-frame to the test polypeptide, which varies among the cells in the
library, that is operably linked to a second DNA segment. The second DNA segment contains
a promoter, an RNA recognition sequence foreign to the anti-terminator protein, a transcription
termination site and a reporter gene. The termination site blocks transcription of the reporter

30 gene in the absence of a protein with anti-termination activity and affinity for the RNA
recognition sequence. If the test polypeptide has specific affinity for the recognition sequence,
it binds via the polypeptide to the RNA recognition sequence of a transcript from the second

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DNA segment thereby inducing transcription of the second DNA segment to proceed through

the termination site to the reporter gene resulting in expression of the reporter gene.

U.S. Patent No. 5,859,227 describes methods for identifying possible binding sites for

RNA binding proteins in nucleic acid molecules, and confirming the identity of such

5   prospective binding sites by detection of interaction between the prospective binding site and

RNA binding proteins. These methods involve identification of possible binding sites for RNA

binding proteins, by either searching databases for untranslated regions of gene sequences or

cloning untranslated sequences using a single specific primer and an universal primer, followed

by confirmation that the untranslated regions in fact interact with RNA binding proteins using

10   the RNA/RBP detection assay. Genomic nucleic acid can further be screened for putative

binding site motifs in the nucleic acid sequences. Information about binding sites that are

confirmed in the assay then can be used to redefine or redirect the nucleic acid sequence search

criteria, for example, by establishing or refining a consensus sequence for a given binding site

motif.

15   SenGupta, et al., Proc. Nat. Acad. Sci. U.S.A., 93:8496-8501 (1996) describes a yeast

genetic method to detect and analyze RNA-protein interactions in which the binding of a

bifunctional RNA to each of two hybrid proteins activates transcription of a reporter gene in

vivo (see also Wang, et al., Genes & Dev., 10:3028-3040 (1996)). SenGupta, et al. demonstrate

that this three-hybrid system enables the rapid, phenotypic detection of specific RNA-protein

20   interactions. As examples, SenGupta, et al. use the binding of the iron regulatory protein 1

(IRP1) to the iron response element (IRE), and of HIV trans-activator protein (Tat) to the HIV

trans-activation response element (TAR) RNA sequence. The three-hybrid assay relies only on

the physical properties of the RNA and protein, and not on their natural biological activities; as

a result, it may have broad application in the identification of RNA-binding proteins and RNAs,

25   as well as in the detailed analysis of their interactions.

The following Table 6 illustrates certain properties of several RNA binding

sequence/RNA sequence pair systems.

Table 6.   Examples of RNA binding sequence/RNA sequence pairs

RNA binding sequence	RNA binding sequence motif	RNA sequence	Reference (U.S. Patent No.)
BINDR	double-stranded RNA-binding	double-stranded RNA poly(rI) and poly (rC)	5,858,675
Protein extract from SH-SY5Y cells	5' untranslated region (UTR)	UTR of Glut1 (SEQ ID NO. 81); 5' UTR	5,859,227

		of (HMG,CoA Red) (SEQ ID NO. 82); 5' UTR of human C4b-binding α chain (SEQ ID NO. 83); 5' UTR of human CD45 (SEQ ID NO. 84)	

3)   Preparation of nucleic acid binding proteins

Preparation of nuclear and cytoplasmic extracts

Extracts prepared from the isolated nuclei of cultured cells are functional in accurate *in vitro* transcription and mRNA processing (See generally, *Current Protocols in Molecular Biology* (1998) § 12.1., John Wiley & Sons, Inc.). Thus, such extracts can be used directly for functional studies and as the starting material for purification of the proteins involved in these processes. To prepare nuclear extracts, tissue culture cells are collected, washed, and suspended in hypotonic buffer. The swollen cells are homogenized and nuclei are pelleted. The cytoplasmic fraction is removed and saved, and nuclei are resuspended in a low-salt buffer. Gentle dropwise addition of a high-salt buffer then releases soluble proteins from the nuclei (without lysing the nuclei). Following extraction, the nuclei are removed by centrifugation, the nuclear extract supernatant is dialyzed into a moderate salt solution, and any precipitated protein is removed by centrifugation.

The nuclear and cytoplasmic extraction procedure (see, *e.g.*, Dignam, *et al.*, 1983, *Nucl. Acids. Res.* 11:1475-1489 (Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei); Dignam, *et al.*, 1983, *Methods Enzymol.* 101:582-598 (Eukaryotic gene transcription with purified components); Krainer, *et al.*, 1984, *Cell* 36:993-1005 (Normal and mutant human β-globin pre-mRNAs are faithfully and efficiently spliced in vitro); Lue, *et al.*, 1987, *Proc. Natl. Acad. Sci. U.S.A.* 84:8839-8843 (Accurate initiation at RNA polymerase II promoters in extracts from *Saccharomyces cerevisiae*); Manley, *et al.*, 1980, *Proc. Natl. Acad. Sci. U.S.A.* 77:3855-3859 (DNA-dependent transcription of adenovirus genes in a soluble whole-cell extract); Weil, *et al.*, 1979, *J. Biol. Chem.* 254:6163-6173 (Faithful transcription of eukaryotic genes by RNA polymerase III in systems reconstituted with purified DNA templates); and Weil, *et al.*, 1979, *Cell* 18:469-484 (Selective and accurate initiation of transcription at the Ad2 major late promotor in a soluble system dependent on

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purified RNA polymerase II and DNA)) and the identified protein-DNA interaction pairs can be used in the present system.

4)   Assays for identifying nucleic acid binding proteins

a.   Mobility shift DNA-binding assay

5   The DNA-binding assay using nondenaturing polyacrylamide gel electrophoresis (PAGE) provides a simple, rapid, and extremely sensitive method for detecting sequence-specific DNA-binding proteins (See generally, *Current Protocols in Molecular Biology* (1998) § 12.2., John Wiley & Sons, Inc.). Proteins that bind specifically to an end-labeled DNA fragment retard the mobility of the fragment during electrophoresis, resulting in discrete bands

10   corresponding to the individual protein-DNA complexes. The assay can be used to test binding of purified proteins or of uncharacterized factors found in crude extracts. This assay also permits quantitative determination of the affinity, abundance, association rate constants, dissociation rate constants, and binding specificity of DNA-binding proteins.

b.   Basic mobility shift assay procedure

15   The basic mobility shift assay procedure includes 4 steps: (1) preparation of a radioactively labeled DNA probe containing a particular protein binding site; (2) preparation of a nondenaturing gel; (3) a binding reaction in which a protein mixture is bound to the DNA probe; and (4) electrophoresis of protein-DNA complexes through the gel, which is then dried and autoradiographed. The mobility of the DNA-bound protein is retarded while that of the

20   non-bound protein is not retarded.

c.   Competition mobility shift assay

One important aspect of the mobility shift DNA-binding assay is the ease of assessing the sequence specificity of protein-DNA interactions using a competition binding assay. This is necessary because most protein preparations will contain specific and nonspecific DNA

25   binding proteins. For a specific competitor, the same DNA fragment (unlabeled) as the probe can be used. The nonspecific competitor can be essentially any fragment with an unrelated sequence, but it is useful to roughly match the probe and specific competitor for size and configuration of the ends. For example, some proteins bind blunt DNA ends nonspecifically. These would not be competed by circular plasmid or a fragment with overhands, leading to the

30   false conclusion that the protein-DNA complex represented specific binding. Perhaps the best

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control competitor is a DNA fragment that is identical to the probe fragment except for a mutation(s) in the binding site that is known to disrupt function (and presumably binding).

d.   Antibody supershift assay

Another useful variation of the mobility shift DNA-binding assay is to use antibodies to
5   identify proteins present in the protein-DNA complex. Addition of a specific antibody to a binding reaction can have one of several effects. If the protein recognized by the antibody is not involved in complex formation, addition of the antibody should have no effect. If the protein that forms the complex is recognized by the antibody, the antibody can either block complex formation, or it can form an antibody-protein-DNA ternary complex and thereby
10   specifically result in a further reduction in the mobility of the protein-DNA complex (supershift). Results may be different depending upon whether the antibody is added before or after the protein binds DNA (particularly if there are epitopes on the DNA-binding surface of the protein).

The mobility shift DNA-binding assay has been successfully employed (see, *e.g.*,
15   Carthew, *et al.*, 1985, *Cell* 43:439-448 (An RNA polymerase II transcription factor binds to an upstream element in the adenovirus major late promoter); Chodosh, *et al.*, 1986, *Mol. Cell. Biol.* 6:4723-4733 (A single polypeptide possesses the binding and activities of the adenovirus major late transcription factor); Fried, *et al.*, 1981, *Nucl. Acids. Res.*, 9:6505-6525 (Equilibria and kinetics of *lac* repressor-operator interactions by polyacrylamide gel electrophoresis);
20   Fried, *et al.*, 1984, *J. Mol. Biol.* 172:241-262 (Kinetics and mechanism in the reaction of gene regulatory proteins with DNA); Fried, *et al.*, 1984, *J. Mol. Biol.* 172:263-282 (Equilibrium studies of the cyclic AMP receptor protein-DNA interaction); Garner, *et al.*, 1981, *Nucl. Acids Res.* 9:3047-3060 (A gel electrophoresis method for quantifying the binding of proteins to specific DNA regions: Application to components of the *Escherichia coli* lactose operon
25   regulatory system); Hendrickson, *et al.*, 1984, *J. Mol. Biol.* 174:611-628 (Regulation of the *Escherichia coli* L-arabinose operon studied by gel electrophoresis DNA binding assay); Kristie, *et al.*, 1986, *Proc. Natl. Acad. Sci. U.S.A.* 83:3218-3222 (The major regulatory protein of herpes simplex virus type 1, is stably and specifically associated with promoter-regulatory domains of a genes and/or selected viral genes); Lieberman, *et al.*, 1994, *Genes & Dev.* 8:995-
30   1006 (A mechanism for TAFs in transcriptional activation: Activation domain enhancement of TFIID-TFIIA-promoter DNA complex formation); Riggs, *et al.*, 1970, *J. Mol. Biol.* 48:67-83 (*Lac* repressor-operator interactions: I. Equilibrium studies); Singh, *et al.*, 1986, *Nature*

319:154-158 (A nuclear factor that binds to a conserved sequence motif in transcriptional control elements of immunoglobulin genes); Staudt, *et al.*, 1986, *Nature* 323:640-643 (A lymphoid-specific protein binding to the octamer motif of immunoglobulin genes); Strauss, *et al.*, 1984, *Cell* 37:889-901 (A protein binds to a satellite DNA repeat at three specific sites that

5 would be brought into mutual proximity by DNA folding in the nucleosome); and Zinkel, *et al.*, 1987, *Nature* 328:178-181 (DNA bend direction by phase-sensitive detection)) and the identified protein-DNA interaction pairs can be used in the present system.

e. Methylation and uracil interference assay

Interference assays identify specific residues in the DNA binding site that, when

10 modified, interfere with binding of the protein (See generally, *Current Protocols in Molecular Biology* (1998) § 12.3., John Wiley & Sons, Inc.). These protocols use end-labeled DNA probes that are modified at an average of one site per molecule of probe. These probes are incubated with the protein of interests, and protein-DNA complexes are separated from free probe by the mobility shift assay. A DNA probe that is modified at a position that interferes

15 with binding will not be retarded in this assay; thus, the specific protein-DNA complex is depleted for DNA that contains modifications on bases important for binding. After gel purification the bound and unbound DNA are specifically cleaved at the modified residues and the resulting products analyzed by electrophoresis on polyacrylamide sequencing gels and autoradiography. These procedures provide complementary information about the nucleotides

20 involved in protein-DNA interactions.

1) Methylation interference assays

In methylation interference, probes are generated by methylating guanines (at the N-7 position) and adenines (at the N-3 position) with DMS; these methylated bases are cleaved specifically by piperidine. Methylation interference identifies guanines and adenines in the

25 DNA binding site that, when methylated, interfere with binding of the protein. The protocol uses a single end-labeled DNA probe that is methylated at an average of one site per molecule of probe. The labeled probe is a substrate for a protein-binding reaction. DNA-protein complexes are separated from the free probe by the mobility shift DNA-binding assay. A DNA probe that is methylated at a position that interferes with binding will not be retarded in this

30 assay. Therefore, the specific DNA-protein complex is depleted for DNA that contains methyl groups on purines important for binding. After gel purification, DNA is cleaved with

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piperidine. Finally, these fragments are electrophoresed on polyacrylamide sequencing gels and autoradiographed. Guanines and adenines that interfere with binding are revealed by their absence in the retarded complex relative to a lane containing piperidine-cleaved free probe. This procedure offers a rapid and highly analytical means of characterizing DNA-protein

5   interactions.

2)   Uracil interference assay

In uracil interference, probes are generated by PCR amplification in the presence of a mixture of TTP and dUTP, thereby producing products in which thymine residues are replaced by deoxyuracil residues (which contains hydrogen in place of the thymine 5-methyl group).

10   Uracil bases are specifically cleaved by uracil-N-glycosylase to generate apyrimidinic sites that are susceptible to piperidine. Uracil interference identifies thymines in a DNA binding site that, when modified, interfere with binding of the protein. Probes generated by PCR amplification in the presence of TTP and dUTP incorporate deoxyuracil in place of thymine residues. PCR products are incubated with the binding protein and resulting complexes are

15   separated from unbound DNA. The DNA recovered from the protein-DNA complex is treated with uracil-N-glycosylase and piperidine, and the products are then electrophoresed on a denaturing polyacrylamide gel.

The methylation and uracil interference assays have been successfully used (see, *e.g.*, Baldwin, *et al.*, 1988, *Proc. Natl. Acad. Sci. U.S.A.* 85:723-727 (Two transcription factors,

20   H2TF1 and NF-kB, interact with a single regulatory sequence in the class I MHC promoter); Brunelle, *et al.*, 1987, *Proc. Natl. Acad. Sci. U.S.A.* 84:6673-6676 (Missing contact probing of DNA-protein interactions); Goeddel, *et al.*, 1978, *Proc. Natl. Acad. Sci. U.S.A.* 75:3579-3582 (How lac repressor recognizes lac operator); Ivarie, *et al.*, 1987, *Nucl. Acids Res.* 15:9975-9983 (Thymine methyls and DNA-protein interactions); Maxam, *et al.*, 1980, *Methods Enzymol*

25   65:499-560 (Sequencing end-labeled DNA with base-specific chemical cleavages); Pu, *et al.*, 1992, *Nucl. Acids Res.* 20:771-775 (Uracil interference, a rapid and general method for defining protein-DNA interactions involving the 5-methyl group of thymines: The GCN4-DNA complex); Siebenlist, *et al.*, 1980, *Proc. Natl. Acad. Sci. U.S.A.* 77:122-126 (Contacts between *E. coli* RNA polymerase and an early promoter of phase T7); and Hendrickson, *et al.*,

30   1985, *Proc. Natl. Acad. Sci. U.S.A.* 82:3129-3133 (A dimer of AraC protein contacts three adjacent major groove regions at the Ara I DNA site)) and the identified protein-DNA interaction pairs can be used in the present system.

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3)   DNase I footprint analysis

Deoxyribonuclease I (DNase I) protection mapping, or footprinting, is a valuable technique for locating the specific binding sites of proteins on DNA (See generally, *Current Protocols in Molecular Biology* (1998) § 12.4., John Wiley & Sons, Inc.). The basis of this assay is that bound protein protects that phosphodiester backbone of DNA from DNase I catalyzed hydrolysis. Binding sites are visualized by autoradiography of the DNA fragments that result form hydrolysis, following separation by electrophoresis on ᵈᵉⁿᵃᵗᵘʳing DNA sequencing gels. Footprinting has been developed further as a quantitative technique to determine separate binding curves for each individual protein-binding site on the DNA. For each binding site, the total energy of binding is determined directly from that site's binding curve. For sites that interact cooperatively, simultaneous numerical analysis of all the binding curves can be used to resolve the intrinsic binding and cooperative components of these energies.

DNase I footprint analysis has been successfully employed (see, *e.g.*, Ackers, *et al.*, 1982, *Proc. Natl. Acad. Sci. U.S.A.* 79:1129-1133 (Quantitative model for gene regulation by lambda phage repressor); Ackers, *et al.*, 1983, *J. Mol. Biol.* 170:223-242 (Free energy coupling within macromolecules: The chemical work of ligand binding at the individual sites in cooperative systems); Brenowitz, *et al.*, 1986, *Proc. Natl. Acad. Sci. U.S.A.* 83:8462-8466 (Footprint titrations yield valid thermodynamic isotherms.); Brenowitz, *et al.*, 1986, *Meth. Enzymol.* 130:132-181 (Quantitative DNase I footprint titration: A method for studying protein-DNA interactions); Dabrowiak, *et al.*, 1989, *In Chemistry and Physics of DNA-Ligand Interactions* (N.R. Kallenback, ed.) Adenine Press. (Quantitative footprinting analysis of drug-DNA interactions); Galas, *et al.*, 1978, *Nucl. Acids Res.* 5:3157-3170 (DNase footprinting: A simple method for the detection of protein-DNA binding specificity); Hertzberg, *et al.*, 1982, *J. Am. Chem. Soc.* 104:313-315 (Cleavage of double helical DNA by (methidiumpropyl-EDTA) iron (II)); Johnson, *et al.*, 1979, *Proc. Natl. Acad. Sci. U.S.A.* 76:5061-5065 (Interactions between DNA-bound repressors govern regulation by the lambda phage repressor); Johnson, *et al.*, 1985, *Meth. Enzymol.* 117:301-342 (Nonlinear least-squares analysis); Senear, *et al.*, 1986, *Biochemistry* 25:7344-7354 (Energetics of cooperative protein-DNA interactions: Comparison between quantitative DNase I footprint titration and filter binding); and Tullius, *et al.*, 1987, *Meth. Enzymol.* 155:537-558 (Hydroxyl radical footprinting: A high resolution method for

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mapping protein-DNA contacts), and the identified protein-DNA interaction pairs can be used in the present system.

4)   Screening a λgt11 expression library with recognition-site DNA

5   A clone encoding a sequence-specific protein can be detected in a λgt11 library because its recombinant protein binds specifically to a radiolabeled recognition-site DNA (See generally, *Current Protocols in Molecular Biology* (1998) § 12.7., John Wiley & Sons, Inc.). Bacteriophage from a cDNA library constructed in the vector λgt11 are plated under lytic growth conditions. After plaques appear, expression of the β-galactosidase fusion proteins

10   encoded by the recombinant phage is induced by placing nitrocellulose filters impregnated with IPTG onto the plate. Phage growth is continued and is accompanied by the immobilization of proteins, from lysed cells, onto the nitrocellulose filters. The filters are lifted after this incubation, blocked with protein, then reacted with a radiolabeled recognition-site DNA (containing one or more binding sites for the relevant sequence-specific protein) in the presence

15   of an excess of nonspecific competitor DNA. After the binding reaction, the filters are washed to remove nonspecifically bound probe and processed for autoradiography. Potentially positive clones detected in the primary screen are rescreened after a round of plaque purification. Recombinants which screen positively after enrichment and whose detection specifically requires the recognition-site probe (non detected with control probes lacking the recognition

20   site for the relevant protein) are then isolated by further rounds of plaque purification.

        The λgt11 expression screening methods have been successfully used (see, *e.g.*, Androphy, *et al.*, 1987, *Nature (Lond.)* 325:70-73 (Bovine papillomavirus E2 trans-activating gene product binds to specific sites in papillomavirus DNA); Arndt, *et al.*, 1986, *Proc. Natl. Acad. Sci. U.S.A.* 83:8516-8520 (GCN4 protein, a positive transcription factor in yeast, binds

25   general control promoters at 5'TGACTC3' sequences); Chodosh, *et al.*, 1988, *Cell* 53:25-35 (A yeast and a human CCAAT-binding protein have heterologous subunits that are functionally interchangeable); Desplan, *et al.*, 1985, *Nature (Lond.)* 318:630-635 (The *Drosophila* developmental gene, engrailed, encodes a sequence-specific DNA binding activity); Hoeffler, *et al.*, 1988, *Science* 242:1430-1433 (Cyclic AMP-responsive DNA-binding protein: Structure

30   based on a cloned placental cDNA); Hsiou-Chi, *et al.*, 1988, *Science* 242:69-71 (Distinct cloned class II MHC DNA binding proteins recognize the X box transcription element); Ingraham, *et al.*, 1988, *Cell* 55:519-529 (A tissue-specific transcription factor containing a

homeo domain specifies a pituitary phenotype); Kadonaga, *et al.*, 1987, *Cell* 51:1079-1090

(Isolation of cDNA encoding transcription factor Sp1 an functional analysis of the DNA

binding domain); Keegan, *et al.*, 1986, *Science* 231:699-704 (Separation of DNA binding from

the transcription-activating function of a eukaryotic regulatory protein); Miyamoto, *et al.*, 1988,

5   *Cell* 54:903-913 (Regulated expression of a gene encoding a nucleic factor, IRF-1, that

specifically binds to IFN-β gene regulatory elements); Murre, *et al.*, 1989, *Cell* 56:777-783 (A

new DNA binding and dimerization motif in immunoglobulin enhancer binding, daughterless,

MyoD and myc proteins); Müller, *et al.*, 1988, *Nature (Lond.)* 336:544-551 (A cloned octamer

transcription factor stimulates transcription from lymphoid specific promoters in non-B cells);

10   Rawlins, *et al.*, 1985, *Cell* 42:859-868 (Sequence-specific DNA binding of the Epstein-Barr

viral nuclear antigen (EBNA-1) to clustered sites in the plasmid maintenance region); Reith, *et

al.*, 1989, *Proc. Natl. Acad. Sci. U.S.A.* 86:4200-4204 (Cloning of the major histocompatibility

complex class II promoter affected in a hereditary defect in class II gene regulation); Singh, *et

al.*, 1988, *Cell* 52:415-423 (Molecular cloning of an enhancer binding protein: Isolation by

15   screening of an expression library with a recognition site); Staudt, *et al.*, 1988, *Science*

241:577-580 (Molecular cloning of a lymphoid-specific cDNA encoding a protein that binds to

the regulatory octamer DNA motif); Sturm, *et al.*, 1988, *Genes & Dev.* 2:1582-1599 (The

ubiquitous octamer protein Oct-1 contains a Pou domain with a homeo subdomain); Vinson, *et

al.*, 1988, *Genes & Dev.* 2:801-806 (*In situ* detection of sequence-specific DNA binding

20   activity specified by a recombinant bacteriophage); Weinberger, *et al.*, 1985, *Science* 228:740-

742 (Identification of human glucocorticoid receptor complementary DNA clones by epitope

selection); and Young, *et al.*, 1983, *Science* 222:778-782 (Yeast RNA polymerase II genes:

Isolation with antibody probes)) and the identified protein-DNA interaction pairs can be used in

the present system.

25   **5)   Rapid separation of protein-bound DNA from
                                free DNA**

This method relies on the ability of nitrocellulose to bind proteins but not double-

stranded DNA (See generally, *Current Protocols in Molecular Biology* (1998) § 12.8., John

Wiley & Sons, Inc.). Use of radioactively labeled double-stranded DNA fragments allows

30   quantitation of DNA bound to the protein at various times and under various conditions,

permitting kinetic and equilibrium studies of DNA-binding interactions. Purified protein is

mixed with double-stranded DNA in an appropriate buffer to allow interaction. After

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incubation, the mixture is suction filtered through nitrocellulose, allowing unbound DNA to pass through the filter while the protein (and any DNA interacting with it) is retained.

Nitrocellulose filter methods have been successfully used (see, e.g., Barkley, et al., 1975, *Biochemistry* 14:1700-1712 (Interaction of effecting ligands with *lac* repressor and
5   repressor-operator complex); Fried, et al., 1981, *Nucl. Acids Res.* 9:6505-6525 (Equilibria and kinetics of *lac* repressor-operator interactions by polyacrylamide gel electrophoresis); Hinkle, et al., 1972, *J. Mol. Biol.* 70:157-185 (Studies of the binding of *Escherichia coli* RNA polymerase to DNA I. The role of sigma subunit in site selection); Hinkle, et al., 1972, *J. Mol. Biol.* 70:187-195 (Studies of the binding of *Escherichia coli* RNA polymerase to DNA II. The
10   kinetics of the binding reaction); Hinkle, et al., 1972, *J. Mol. Biol.* 70:197-207 (Studies of the binding of *Escherichia coli* RNA polymerase to DNA III. Tight binding of RNA polymerase holoenzyme to single-strand breaks in T7 DNA); Jones, et al., 1966, *J. Mol. Biol.* 22:199-209 (Studies on the binding of RNA polymerase to polynucleotides); Lin, et al., 1972, *J. Mol. Biol.* 72:671-690 (*Lac* repressor binding to non-operator DNA: Detailed studies and a comparison of
15   equilibrium and rate competition methods); Lin, et al., 1975, *Cell* 4:107-111 (The general affinity of *lac* repressor for *E. coli* DNA: Implications for gene regulation in procaryotes and eucaryotes); Nirenberg, et al., 1964, *Science* 145:1399-1407 (RNA codewords and protein synthesis: The effect of trinucleotides upon the binding of sRNA to ribosomes); Ptashne, et al., 1987, A Genetic Switch: Gene Control and Phage λ pp. 80-83 and 109-118. Cell Press,
20   Cambridge, MA and Blackwell Scientific, Boston, MA; Riggs, et al., 1970, *J. Mol. Biol.* 48:67-83 (*Lac* repressor-operator interactions: I. Equilibrium studies); Strauss, et al., 1980, *Biochemistry* 19:3496-3504 (Binding of *Escherichia coli* ribonucleic acid polymerase holoenzyme to a bacteriophage T7 promoter-containing fragment: Selectivity exists over a wide range of solution conditions); Strauss, et al., 1980, *Biochemistry* 19:3504-3515 (Binding
25   of *Escherichia coli* ribonucleic acid polymerase holoenzyme to a bacteriophage T7 promoter-containing fragment: Evaluation of promoter binding constants as a function of solution conditions); and Strauss, et al., 1981, *Gene* 13:75-87 (Variables affecting the selectivity and efficiency of retention of DNA fragments by *E. coli* RNA polymerase in the nitrocellulose-filter binding assay)) and the identified protein-DNA interaction pairs can be used in the
30   present system.

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f.   Lipid binding moieties

The conjugate can also contain a lipid binding protein, peptide or effective fragment thereof. Its specific binding partner can be lipids generally, a set of lipids or a particular lipid. Any lipid binding moiety, particularly proteins, peptides or effective fragments thereof can be

5   used in the present system. For example, the lipid binding protein can bind to a triacylglycerol, a wax, a phosphoglyceride, a sphingolipid, a sterol and a sterol fatty acid ester. More preferably, the lipid binding sequence comprises a C2 motif or an amphipathic α-helix motif.

Any lipid binding sequence/lipid pair can be designed, screened or selected according to the methods known in the art (see, *e.g.*, Kane, *et al.*, *Anal. Biochem.*, 233(2):197-204 (1996);

10   Arnold, *et al.*, *Biochim. Biophys. Acta*, 1233(2):198-204 (1995); Miller and Cistola, *Mol. Cell. Biochem.*, 123(1-2):29-37 (1993); and Teegarden, *et al.*, *Anal. Biochem.*, 199(2):293-9 (1991).

For example, Kane, *et al.*, *Anal. Biochem.*, 233(2):197-204 (1996) describes that the fluorescent probe 1-anilinonapthalene 8-sulfonic acid (1,8-ANS) has been used to characterize a general assay for members of the intracellular lipid-binding protein (iLBP) multigene family.

15   The adipocyte lipid-binding protein (ALBP), the keratinocyte lipid-binding protein (KLBP), the cellular retinol-binding protein (CRBP), and the cellular retinoic acid-binding protein I (CRABPI) have been characterized as to their ligand binding activities using 1,8-ANS. ALBP and KLBP exhibited the highest affinity probe binding with apparent dissociation constants (Kd) of 410 and 530 nM, respectively, while CRBP and CRABPI bound 1,8-ANS with

20   apparent dissociation constants of 7.7 and 25 microM, respectively. In order to quantitate the fatty acid and retinoid binding specificity and affinity of ALBP, KLBP, and CRBP, a competition assay was developed to monitor the ability of various lipid molecules to displace bound 1,8-ANS from the binding cavity. Oleic acid and arachidonic acid displaced bound 1,8-ANS from ALBP, with apparent inhibitor constants (Ki) of 134 nM, while all-trans-retinoic

25   acid exhibited a seven-fold lower Ki (870 nM). The short chain fatty acid octanoic acid and all-trans-retinol did not displace the fluorophore from ALBP to any measurable extent. In comparison, the displacement assay revealed that KLBP bound oleic acid and arachidonic acid with high affinity (Ki = 420 and 400 nM, respectively) but bound all-trans-retinoic acid with a markedly reduced affinity (Ki = 3.6 microM). Like that for ALBP, neither octanoic acid nor

30   all-trans-retinol were bound by KLBP. Displacement of 1,8-ANS from CRBP by all-trans-retinal and all-trans-retinoic acid yielded Ki values of 1.7 and 5.3 microM, respectively. These results indicate the utility of the assay for characterizing the ligand binding characteristics of

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members of the iLBP family and suggests that this technique may be used to characterize the ligand binding properties of other hydrophobic ligand binding proteins.

Arnold, et al., Biochim. Biophys. Acta, 1233(2):198-204 (1995) describes an assay for analyzing the specific binding of proteins to lipid ligands contained within vesicles or micelles.

5   This assay, referred to as the electrophoretic migration shift assay, was developed using a model system composed of cholera toxin and of its physiological receptor, monosialoganglioside GM1. Using polyacrylamide gel electrophoresis in non-denaturing conditions, the migration of toxin components known to interact with GM1 was retarded when GM1 was present in either lipid vesicles or micelles. This effect was specific, as the migration

10 of proteins not interacting with GM1 was not modified. The localization of retarded proteins and of lipids on gels was further determined by autoradiography. The stoichiometry of binding between cholera toxin and GM1 was determined, giving a value of five GM1 per one pentameric assembly of cholera toxin B-subunits, in agreement with previous studies. The general applicability of this assay was further established using streptavidin and annexin V

15 together with specific lipid ligands. This assay is fast, simple, quantitative, and requires only microgram quantities of protein.

Miller and Cistola, Mol. Cell. Biochem., 123(1-2):29-37 (1993) teaches that titration calorimetry can be used as a method for obtaining binding constants and thermodynamic parameters for the cytosolic fatty acid- and lipid-binding proteins. A feature of this method is

20 its ability to accurately determine binding constants in a non-perturbing manner. This is acheived because the assay does not require separation of bound and free ligand to obtain binding parameters. Also, the structure of the lipid-protein complex was not perturbed, since native ligands were used rather than non-native analogues. As illustrated for liver fatty acid-binding protein, the method distinguished affinity classes whose dissociation constants differed

25 by an order of magnitude or less. It also distinguished endothermic from exothermic binding reactions, as illustrated for the binding of two closely related bile salts to ileal lipid-binding protein. The main limitations of the method are its relatively low sensitivity and the difficulty working with highly insoluble ligands, such as cholesterol or saturated long-chain fatty acids. The signal-to-noise ratio was improved by manipulating the buffer conditions, as illustrated for

30 oleate binding to rat intestinal fatty acid binding protein.

Teegarden, et al., Anal. Biochem., 199(2):293-9 (1991) describes an assay for measurement of the affinity of serum vitamin D binding protein for 25-hydroxyvitamin D3, 1,25-dihydroxyvitamin D3, and vitamin D3, using uniform diameter (6.4 microns) polystyrene

beads coated with phosphatidylcholine and vitamin D metabolites as the vitamin D donor. The lipid metabolite coated beads have a solid core, and thus all of the vitamin D metabolites are on the bead surface from which transfer to protein occurs. After incubating these beads in neutral buffer for 3 h, essentially no ^{3}H-labeled vitamin D metabolites desorb from this surface.

5   Phosphatidylcholine/vitamin D metabolite-coated beads (1 microM vitamin D metabolite) were incubated with varying concentrations of serum vitamin D binding protein under conditions in which the bead surfaces were saturated with protein, but most of the protein was free in solution. After incubation, beads were rapidly centrifuged without disturbing the equilibrium of binding and vitamin D metabolite bound to sDBP in solution was assayed in the supernatant.

10   All three vitamin D metabolites became bound to serum vitamin D binding protein, and after 10 min of incubation the transfer of the metabolites to serum vitamin D binding protein was time independent. The transfer followed a Langmuir isotherm, and the Kd for each metabolite binding to serum vitamin D binding protein was derived by nonlinear least-squares fit analysis. From this analysis the following values for the Kd were obtained: 5.59 x 10^{-6} M, 25-

15   hydroxyvitamin D; 9.45 x 10^{-6} M, 1,25-dihydroxyvitamin D; and 9.17 x 10^{-5} M, vitamin D. The method disclosed herein avoids problems encountered in previous assays and allows the precise and convenient determination of binding affinities of vitamin D metabolites and serum vitamin D binding protein.

In addition, known protein/lipid binding pairs can be used in the methods and with the

20   products provided herein (see, e.g., Hinderliter, et al., *Biochim. Biophys. Acta*, 1448(2):227-35 (1998) (C2 motif binds phospholipid in a manner that is modulated by Ca2+ and confers membrane-binding ability on a wide variety of proteins, primarily proteins involved in signal transduction and membrane trafficking events); Campagna, et al., *J. Diary Sci.*, 81(12):3139-48 (1998) (an amphipathic helical lipid-binding motif of a glycosylated phosphoprotein,

25   component PP3 in bovine milk); Chae, et al., *J. Biol. Chem.*, 273(40):25659-63 (1998) (The C2A domain of synaptotagmin I, which binds Ca2+ and anionic phospholipids); Johnson, et al., *Biochemistry*, 37(26):9509-19 (1998) (the membrane binding domain of phosphocholine cytidylyltransferase (CT) includes a continuous amphipathic alpha-helix between residues approximately 240-295 anionic lipids); Kiyosue, et al., *Plant Mol. Biol.*, 35(6):969-72 (1997)

30   (Ca2+-dependent lipid-binding domains of cytosolic phospholipase A2, protein kinase C, Rabphilin-3A, and Synaptotagmin 1 of animals); Welters, et al., *Proc. Natl. Acad. Sci. USA*, 91(24):11398-402 (1994) (calcium-dependent lipid-binding domain is near the N terminus of phosphatidylinositol (PI) 3-kinase cloned from Arabidopsis thaliana); and Filoteo, et al., *J.*

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Biol. Chem., 267(17):11800-5 (1992) (Peptide G25:

LysLysAlaValLysValProLysLysGluLysSerValLeuGlnGlyLysLeuThrArgLeuAlaValGlnIle (SEQ ID

No. 85)) representing the putative lipid-binding region (G region) of the erythrocyte Ca2+

pump interacted with acidic lipids, as shown by the increase in size of phosphatidylserine

5   liposomes in its presence)).

g.   Polysaccharide binding moieties

The conjugate can include a polysaccharide binding protein, peptide or effective

fragment thereof. Its specific binding partner can be polysaccharides generally, a set of

polysaccharides or a particular polysaccharide. Any polysaccharide binding moiety, such as a

10   protein, can be used in the present system and include but are not limited to a polysaccharide

binding sequence that binds to starch, glycogen, cellulose or hyaluronic acid.

Any polysaccharide binding protein/polysaccharide pair can be designed, screened or

selected according to the methods known in the art including the methods disclosed in Kuo, *et

al., J. Immunol. Methods*, 43(1):35-47 (1981); and Brandt, *et al., J. Immunol.*, 108(4):913-20

15   (1972) (a radioactive antigen-binding assay for *Neisseria meningitidis* polysaccharide

antibody). Kuo, *et al., J. Immunol. Methods*, 43(1):35-47 (1981) provides a polyethylene

glycol (PEG) radioimmunoprecipitation assay for the detection of antibody to *Haemophilus

influenza* b capsular polysaccharide, polyribosylribitol phosphate (PRP). The radioactive

antigen, [^{3}H]PRP, with a high specific activity, was produced by growing the organism in the

20   presence of [^{3}H]ribose and was purified by hydroxylapatite and Sepharose^{TM} 4B column

chromatography. In the assay, PEG (12.5%) was used to separate antibody-bound [^{3}H]PRP

from free [^{3}H]PRP. The assay covered the range of 0.5 and 20 ng antibody/assay at a

maximum sensitivity of 0.5 approximately 1.0 ng antibody/assay. With various dilutions (1-20

ng antibody/assay) of S. Klein reference antiserum, the within-run coefficient of variation (CV)

25   of 10 replicates ranged from 3.5 to 8.5%. Average CVs of 8.9% and 11.0% were obtained in

the between-run and day-to-day reproducibility studies. The binding of [^{3}H]PRP to S. Klein

reference antiserum was severely inhibited by a minute amount of non-radioactive PRP;

however, no significant interference was found in the presence of high concentrations of

polysaccharides from *Escherichia coli* K100 and *Streptococcus* pneumoniae indicating that the

30   RIA was highly specific for antibody to *H. influenza* b PRP.

In addition, known protein/polysaccharide binding pairs can be used in the methods and

with the products provided herein (see, *e.g.*, Yamaguchi, *et al., Oral Microbiol. Immunol.*,

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13(6):348-54 (1998) (capsule-like serotype-specific polysaccharide antigen lipopolysaccharide
from Actinobacillus actinomycetemcomitans/human complement-derived opsonins); Lucas, *et al.*, *J. Immunol.*, 161(7):3776-80 (1998) (kappa II-A2 light chain CDR-3 junctional residues in
human antibody/Haemophilus influenza type b polysaccharide); Miller, *et al.*, *Carbohydr. Res.*,

5   309(3):219-26 (1998) (fragments of the Shigella dysenteriae type 1 O-specific
polysaccharide/monoclonal IgM 3707 E9); Prehm, *et al.*, *Protein Expr. Purif.*, 7(4):343-6
(1996) (digitonin/hyaluronate synthase); Jiang, *et al.*, *Infect. Immun.*, 63(7):2537-40 (1995)
(mannose-binding protein/Klebsiella O3 lipopolysaccharide); Pelkonen, *et al.*, *J. Bacteriol.*,
174(23):7757-61 (1992) (bacteriophage depolymerase/bacterial polysaccharide); Morishita, *et*

10   *al.*, *Biochem. Biophys. Res. Commun.*, 176(3):949-57 (1991) (Microbial polysaccharide, HS-
142-1/guanylyl cyclase-containing receptor); Ohtomo, *et al.*, *Can. J. Microbiol.*, 36(3):206-10
(1990) (staphylococcal cell surface polysaccharide/human fibrinogen); Yamagishi, *et al.*, *FEBS
Lett.*, 225(1-2):109-12 (1987) (heparin or dermatan sulfate/thrombin); DeAngelis, *et al.*, *J. Biol.
Chem.*, 262(29):13946-52 (1987) (sulfated fucans/bindin, the adhesive protein from sea urchin

15   sperm); Volanakis, *et al.*, *Mol. Immunol.*, 20(11):1201-7 (1983) (human C4/C-reactive protein-
pneumococcal C-polysaccharide complexes); Naruse, *et al.*, *J. Biochem. (Tokyo)*, 90(3):581-7
(1981) (a polysaccharide from the cortex of sea urchin egg/microtubule-associated proteins);
Levy, *et al.*, *J. Exp. Med.*, 153(4):883-96 (1981) (agaropectin and heparin/human IgG proteins);
Hu, *et al.*, *Biochemistry*, 14(10):2224-30 (1975) (glycogen phosphorylase A/a series of

20   semisynthetic, branched saccharides); Fagerstrom, *Microbiology*, 140(9):2399-407 (1994)
(raw-starch-binding consensus amino acids in the C-terminal part of glucoamylase P); Murata,
et al., *J. Vet. Med. Sci.*, 57(3):419-25 (1995) (C-polysaccharide/C-reactive protein (CRP));
Reason, *et al.*, *Infect. Immun.*, 67(2):994-7 (1999) (Antibodies having light (L) chains encoded
by the kappaII-A2 variable region/Haemophilus influenza type b polysaccharide (Hib PS)).

25   **h.   Metal binding moieties**

        The conjugate can contain a metal binding moiety, such as a metal binding protein,
peptide or effective fragment thereof. The specific binding partner can be metal ions generally,
a set of metal ions or a particular metal ion. Any metal binding moiety is contemplated. For
example, the metal binding sequence can bind to a sodium, a potassium, a magnesium, a

30   calcium, a chlorine, an iron, a copper, a zinc, a manganese, a cobalt, an iodine, a molybdenum,
a vanadium, a nickel, a chromium, a fluorine, a silicon, a tin, a boron or an arsenic ion.

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Any metal binding moiety/metal ion pair can be designed, screened or selected according to the methods known in the art including the methods disclosed in U.S. Patent No. 5,679,548; Kang, et al., Virus Res., 49(2):147-54 (1997); Dealwis, et al., Biochemistry, 34(43):13967-73 (1995); and Hutchens, et al., J. Chromatogr., 604(1):125-32 (1992).

5   U.S. Patent No. 5,679,548 discloses a method for producing a metal binding site in a polypeptide capable of binding a preselected metal ion-containing molecule, the step of inducing mutagenesis of a complementarity determining region (CDR) of an immunoglobulin heavy or light chain gene, wherein said mutagenesis introduces a metal binding site, by amplifying the CDR of said gene by a primer extension reaction using a primer

10   oligonucleotide, said oligonucleotide comprising: a) a 3' terminus and a 5' terminus comprising; b) a nucleotide sequence at said 3' terminus complementary to a first framework region of said heavy or light chain immunoglobulin gene; c) a nucleotide sequence at said 5' terminus complementary to a second framework region of said heavy or light chain immunoglobulin gene; and d) a nucleotide sequence between said 3' terminus and 5' terminus according to the

15   formula; [NNS]$_a$, wherein N is independently any nucleotide, S is G or C, and a is from 3 to about 50, and said 3' and 5' terminal nucleotide sequences having a length of about 6 to 50 nucleotides, and sequences complementary thereto.

U.S. Patent No. 5,679,548 also describes a method for producing a metal binding site in a polypeptide capable of binding a preselected metal ion-containing molecule, the step of

20   inducing mutagenesis of a complementarity determining region (CDR) of an immunoglobulin heavy or light chain gene by amplifying the CDR of said gene by a primer extension reaction using a primer oligonucleotide, said oligonucleotide comprising: a) a 3' terminus and a 5' terminus; b) a nucleotide sequence at said 3' terminus complementary to a first framework region of said heavy or light chain immunoglobulin gene; c) a nucleotide sequence at said 5'

25   terminus complementary to a second framework region of said heavy or light chain immunoglobulin gene; and d) a nucleotide sequence between 3' terminus and 5' terminus according to the formula: -X-[NNK]$_a$-X-[NNK]-X, wherein N is independently any nucleotide, K is G or T, X is a trinucleotide encoding a native amino acid residue coded by said immunoglobulin gene and a is from 3 to about 50, and said 3' and 5' terminal nucleotide

30   sequences having a length of about 6 to 50 nucleotides, and sequences complementary thereto. Preferably, the immunoglobulin to be mutagenized is a human immunoglobulin, the CDR is CDR3, the mutagenizing oligonucleotide has the formula: 5'-GTGTATTATTGTGCGAGA[NNS]$_a$TGGGGCCAAGGGACCACG-3' (SEQ ID No. 86), and

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the preselected metal ion-containing molecule is magnetite, copper(II), zinc(II), lead(II), cerium(III), or iron(III).

Kang, *et al.*, *Virus Res.*, 49(2):147-54 (1997) isolated human papillomavirus (HPV) type 18 E7 gene by polymerase chain reaction (PCR) amplification from tissues of Korean
5   cervical cancer patients and cloned into a plasmid vector, pET-3a, for the expression of recombinant E7 protein (rE7) in *Escherichia coli*. The rE7 protein was purified to the homogeneity and its purity was confirmed by HPLC. The purified protein was analyzed for the metal-binding properties by UV spectroscopy and it was shown that two Cd^{2+} or Zn^{2+} ions bind to one E7 protein by the metal-sulfur ligand formation via two Cys-X-X-Cys motifs in E7
10 protein. When the change of intrinsic fluorescence of tryptophan residue was analyzed for rE7-Zn complex, the blue shift of emission wavelength and the decrease in maximum intensity of emission were observed compared with rE7. These results suggest that Zn^{2+}-bound rE7 has undergone conformational change, in which a tryptophan residue located in the second Cys-X-X-Cys motif was moved into solvent-inaccessible or hydrophobic environment.

15   Dealwis, *et al.*, *Biochemistry*, 34(43):13967-73 (1995) present the refined crystal structures of three different conformational states of the Asp153-->Gly mutant (D153G) of alkaline phosphatase (AP), a metalloenzyme from *Escherichia coli*. The apo state is induced in the crystal over a 3 month period by metal depletion of the holoenzyme crystals. Subsequently, the metals are reintroduced in the crystalline state in a time-dependent reversible manner
20 without physically damaging the crystals. Two structural intermediates of the holo form based on data from a 2 week (intermediate I) and a 2 month soak (intermediate II) of the apo crystals with Mg^{2+} and Zn^{2+} have been identified. The three-dimensional crystal structures of the apo (R = 18.1%), intermediate I (R = 19.5%), and intermediate II (R = 19.9%) of the D153G enzyme have been refined and the corresponding structures analyzed and compared. Large
25 conformational changes that extend from the mutant active site to surface loops, located 20 A away, are observed in the apo structure with respect to the holo structure. The structure of intermediate I shows the recovery of the entire enzyme to an almost native-like conformation, with the exception of residues Asp 51 and Asp 369 in the active site and the surface loop (406-410) which remains partially disordered. In the three-dimensional structure of intermediate II,
30 Asp 51 and Asp 369 are essentially in a native-like conformation, but the main chain of residues 406-408 within the loop is still not fully ordered. The D153G mutant protein exhibits weak, reversible, time dependent metal binding in solution and in the crystalline state.

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Hutchens, *et al.*, *J. Chromatogr.*, 604(1):125-32 (1992) prepared synthetic peptides representing metal-binding protein surface domains from the human plasma metal transport protein known as histidine-rich glycoprotein (HRG) to evaluate biologically relevant peptide-metal ion interactions. Three synthetic peptides, representing multiples of a 5-residue repeat sequence (Gly-His-His-Pro-His) (SEQ ID No. 87) from within the histidine- and proline-rich region of the C-terminal domain were prepared. Prior to immobilization, the synthetic peptides were evaluated for identity and sample homogeneity by matrix-assisted UV laser desorption time-of-flight mass spectrometry (LDTOF-MS). Peptides with bound sodium and potassium ions were observed; however, these signal intensities were reduced by immersion of the sample probe tip in water. Mixtures of the three different synthetic peptides were also evaluated by LDTOF-MS after their elution through a special immobilized peptide-metal ion column designed to investigate metal ion transfer. It was found that LDTOF-MS to be a useful new method to verify the presence of peptide-bound metal ions.

In addition, the protein/metal binding pairs, which are known (see, *e.g.*, DiDonato, *et al.*, *Adv. Exp. Med. Biol.*, 448:165-73 (1999) (copper/copper binding domain from the Wilson disease copper transporting ATPase (ATP7B)); Buchko, *et al.*, *Biochem Biophis. Res. Commun.*, 254(1):109-13 (1999) (Zn^{2+}/Xenopus laevis nucleotide excision repair protein XPA); Lai, *et al.*, *Biochemistry*, 37(48):7005-15 (1998) (Zn^{2+}/hdm2 RING finder domain); Mitterauer, *et al.*, *Biochemistry*, 37(46):16183-91 (1998) (The C2 catalytic domain of adenylyl cyclase contains the second metal ion (Mn2+) binding site); Hess, *et al.*, *Protein Sci.*, 7(9):1970-5 (1998) (Zn^{2+}/Human nucleotide excision repair protein XPA); Goedken, *et al.*, *Proteins*, 33(1):135-43 (1998) (Mg^{2+} and Mn^{2+}/ribonuclease H domain of Moloney murine leukemia virus reverse transcriptase); Chang, *et al.*, *Protein Eng.*, 11(1):41-6 (1998) (beta-domain of metallothionein); Champeil, *et al.*, *J. Biol. Chem.*, 273(12):6619-31 (1998) (cytosolic portion of sarcoplasmic reticulum Ca2+-ATPase); Bavoso, *et al.*, *Biochem. Biophys. Res. Commun.*, 242(2):385-9 (1998) (zinc finger peptide containing the Cys-X2-Cys-X4-His-X4-Cys domain encoded by the Drosophila Fw-element); Gitschier, *et al.*, *Nat. Struct. Biol.*, 5(1):47-54 (1998) (metal-binding domain from the Menkes copper-transporting ATPase); Gadhavi, *FEBS Lett.*, 417(1):145-9 (1997) (Zn^{2+}/ion binding site in the DNA binding domain of the yeast transcriptional activator GAL4); Roehm, *et al.*, *Biochemistry*, 36(33):10240-5 (1997) (Zn^{2+}/RING finger domain of BRCA1); Dalton, *et al.*, *Mol. Cell Biol.*, 17(5):2781-9 (1997) (metal response element-binding transcription factor 1 DNA binding involves zinc interaction with the zinc finger domain); Essen, *et al.*, *Biochemistry*, 36(10):2753-62 (1997) (Ca^{2+}/A

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ternary metal binding site in the C2 domain of phosphoinositide-specific phospholipase C-delta1); Curtis, *et al.*, *EMBO J.*, 16(4):834:43 (1997) (Zn^{2+}/CCHC metal-binding domain in Nanos); Worthington, *et al.*, *Proc. Natl. Acad. Sci. USA*, 93(24):13754-9 (1996) (zinc-binding domain of Nup475); Mahadevan, *et al.*, *Biochemistry*, 34(7):2095-106 (1995) (Ba^{2+}, Ca^{2+},

5   Mg^{2+}, Mn^{2+}, Ni^{2+}, Zn^{2+}/A divalent metal ion binding site in the kinase insert domain of the alpha-platelet-derived growth factor receptor); Pan, *et al.*, *Biochem. Biophys. Res. Commun.*, 202(1):621-8 (1994) (alpha and beta domains of mammalian metallothionein); Borden, *et al.*, *FEBS Lett.*, 335(2):255-60 (1993) (Cu^{2+}, Zn^{2+}/cysteine/histidine-rich metal binding domain from Xenopus nuclear factor XNF7); Chauhan, *et al.*, *J. Bacteriol.*, 175(22):7222-7 (1993)

10 (Mg^{2+}/Bradyrhizobium japonicum delta-aminolevulinic acid dehydratase is metal-binding domain); Kriegtel, *et al.*, *Biochem. Biophys. Res. Commun.*, 192(2):492-8 (1993) (Zn^{2+}/metal coordination in the human retinoic acid receptor-beta DNA binding domain); Spencer, *et al.*, *Biochem. J.*, 290(1):279-87 (1993) (Co^{2+}, Mg^{2+}, Zn^{2+}/5-aminolaevulinic acid dehydratase from Escherichia coli reactive thiols at the metal-binding domain); Mau, *et al.*, *Protein Sci.*,

15 1(11):1403-12 (1992) (Zn^{2+}/GAL4 DNA-binding domain); Vaughan, *et al.*, *Virology*, 189(1):377-84 (1992) (Zn^{2+}/The herpes simplex virus immediate early protein ICP27 metal binding domain); Boese, *et al.*, *J. Biol. Chem.*, 266(26):17060-6 (1991) (Mg^{2+}/Aminolevulinic acid dehydratase in pea metal-binding domain); Hutchens, *et al.*, *J. Biol. Chem.*, 264(29):17206-12 (1989) (Cu^{2+}, Ni^{2+}, Zn^{2+}/DNA-binding estrogen receptor); Stillman, *et al.*,

20 *Biochem. J.*, 262(1):181-8 (1989) (Cd^{2+} and Zn^{2+}/rabbit liver metallothionein 2); Freedman, *et al.*, *Nature*, 334(6182):543-6 (1988) (Cd^{2+} and Zn^{2+}/metal coordination sites within the glucocorticoid receptor DNA binding domain); Stillman, *et al.*, *J. Biol. Chem.*, 263(13):6128-33 (1988) (Cd^{2+} and Zn^{2+}/metallothionein); and Corson, *et al.*, *Biochemistry*, 25(7):1817-26 (1986) (Ca^{2+}/calcium-binding proteins C-terminal alpha-helix of a helix-loop-helix metal-

25 binding domain)) can be used in the present system.

Among the preferred pairs, are the following metal binding sequence/metal ion pairs (see, U.S. Patent No. 5,679,548) set forth in the following Table 7.

Table 7.   Examples of Metal Ion Binding Sequence/Metal Ion Pairs

Metal Ion	Metal Ion Binding Sequence	SEQ ID NO.
Mg(II)	SerArgArgSerArgHisHisProArgMetTrpAsnGlyLeuAspVal	88
	GlyArgPheLysArgValArgAspArgTrpValValIlePheAspPhe	89
	GlyValAlaArgSerLysLysMetArgGlyLeuTrpArgLeuAspVal	90
	GlyLeuAlaValArgSerLysArgGlyArgPhePheLeuPheAspVal	91
Cu(II)	GlyArgValHisHisHisSerLeuAspVal	92

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Metal Ion	Metal Ion Binding Sequence	SEQ ID NO.
	SerTrpLysHisHisAlaHisTrpAspVal	93
	GlySerTrpAspHisArgGlyCysAspGly	94
	GlyHisHisMetTyrGlyGlyTrpAspHis	95
	GlyHisTrpGlyArgHisSerLeuAspThr	96
	GlyHisIleLeuHisHisGlnLeuAspLeu	97
	SerSerGlnArgLeuMetLeuGlyAspAsn	98
	SerHisHisGlyHisHisTyrLeuAsnHis	99
	GlyLysLeuMetMetSerTrpCysArgAspThrGluGlyCysAspHis	100
	GlyAspThrHisArgGlyHisLeuArgHisHisLeuProHisAspTrp	101
	GlyTrpGlyLeuTrpMetLysProPheValTrpArgAlaTrpAspMet	102
Zn(II)	GlyArgValHisHisHisSerLeuAspVal	103
	SerHisThrHisAlaLeuProLeuAspPhe	104
	GlyGlnSerSerGlyGlyAspThrAspAsp	105
	GlyGlnTrpThrProArgGlyAspAspPhe	106
	GlyArgCysCysProSerSerCysAspGlu	107
	GlyProAlaLysHisArgHisArgHisValGlyGlnMetHisAspSer	108
Pb(III)	GlyAsnLeuArgArgLysThrSerAspIle	109
	GlyGluSerAspSerLysArgGluAspGly	110
	GlyGlyProSerLeuAlaValGlyAspTrp	111
	GlyProLeuGlnHisThrTyrProAspTyr	112
	GlyTrpLysValThrAlaGluAspSerThrGluGlyLeuPheAspLeu	113
	GlyThrArgValTrpArgValCysGlnTrpAsnHisGluGluAspGly	114
	GlyGluTrpTrpCysSerPheAlaMetCysProAlaArgTrpAspPhe	115
	GlyAspThrIlePheGlyValThrMetGlyTyrTyrAlaMetAspVal	116
Ce(III)	GlyGlnValMetGlnGluLeuGlyAspAla	117
	GlyLeuThrGluGlnGlnLeuGlnAspGly	118
	GlyTyrSerTyrSerValSerProAspAla	119
	GlyArgLeuGlyLeuValMetThrAspGlu	120
	SerThrTrpProGlyArgGlnArgLeuGlyGlnAlaLeuSerAspSer	121
	GlyTyrGluLeuSerTrpGlyValAspGlnGlnGluTrpTrpAspIle	122
	GlyProValArgGlyLeuAspGlnSerLysGlyValArgTyrAspAsn	123
	GlyLeuSerGlnHisIleValSerGluThrGlnSerSerGlyAspLeu	124
	GlyLeuGluSerLeuLysValLeuGlyValGlnLeuGlyGlyAspLeu	125
	GlyAsnMetIleLeuGlyGlyProGlyCysTrpSerSerAlaAspIle	126
	GlyCysTrpAsnValGlnArgLeuValValTyrHisProProAspGly	127
	GlyPheGluValThrCysSerTrpPheGlyHisTrpGlyArgAspSer	128
Fe(III)	SerAlaSerMetArgSerAlaIleGlyLeuTrpArgThrMetAspTyr	129

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Metal Ion	Metal Ion Binding Sequence	SEQ ID NO.
	GlyAspArgGluIlePheHisMetGlnTrpProLeuArgValAspVal	130
	SerGlnAsnProGlnGlnValCysGlyValArgCysGlyGlnAspLys	131
	GlyAsnArgLeuSerSerGlyHisLeuLeuLysGlnGlyGlnAspGly	132
	GlyGlySerAspTrpGlnIleGlyAlaCysCysArgGluAspAspLeu	133
	GlyMetValSerMetMetGlyGlnSerArgProThrGlnCysAspCys	134
	GlyValIleLysTrpIleArgArgTrpValArgThrAlaArgAspVal	135
	GlyTrpPheTrpArgLeuLeuProThrProArgAlaProSerAspVal	136

i.   Other facilitating agents

Facilitating agents can be derived from an enzyme, a transport protein, a nutrient or storage protein, a contractile or motile protein, a structural protein, a defense protein, a

5   regulatory protein, or a fluorescent protein. Exemplary of such other fragments are those derived from an enzyme such as a peroxidase, a urease, an alkaline phosphatase, a luciferase and a glutathione S-transferase.

1)   Peroxidase

Any peroxidase can be used in the present system. More preferably, a horseradish

10   peroxidase is used. For example, the horseradish peroxidases with the following GenBank accession Nos. can be used: E01651; D90116 (prxC3 gene); D90115 (prxC2 gene); J05552 (Synthetic isoenzyme C(HRP-C)); S14268 (neutral); OPRHC (C1 precursor); S00627 (C1C precursor); JH0150 (C3 precursor); S00626 (C1B precursor); JH0149 (C2 precursor); CAA00083 (Armoracia rusticana); and AAA72223 (synthetic horseradish perioxidase

15   isoenzyme C (HRP-C)).

2)   urease

Any urease can be used in the present system. For example, the ureases with the following GenBank accession Nos. can be used: AF085729 (Ureaplasma urealyticum serovar); AF056321 (Actinomyces naeslundii); AF095636 (Yersinia pestis); AF006062 (Filobasidiella

20   neoformans var. neoformans (URE1)); U81509 (Coccidioides immitis urease); AF000579 (Bordetella bronchiseptica); U352248 (Streptococcus salivarius); U33011 (Mycobacterium tuberculosis); U89957 (Actinobacillus pleuropneumoniae urease operon (ureABCXEFGD); D14439 (Thermophilic Bacillus); L40490 (Ureaplasma urealyticum T960 urease); L40489 (Ureaplasma urealyticum strain 7); U40842 (Yersinia pseudotuberculosis); M65260 (Canavalia

ensiformis); U29368 (Bacillus pasteurii ure operon); L25079 (Heliobacter heilmannii urease);

L24101 (Yersinia enterocolitica); M31834 (P.mirabilis urease operon); M36068 (K.aerogenes);

L07039 (Klebsiella pneumoniae); M60398 (H.pylori); L03308 (E.coli urease gene cluster);

L03307 (E.coli urease gene cluster).

5   **3)   Alkaline phosphatase**

Any alkaline phosphatase can be used in the present system. For example, the alkaline

phosphatases encoded by nucleic acids with the following GenBank accession Nos. can be

used: AB013386 (Bombyx mori s-Alp soluble alkaline phosphatase); AF154110 (Enterococcus

faecalis (phoZ); M13077 (Human placental); AF052227 (Bos taurus intestinal); AF052226

10   (Bos taurus intestinal); AF079878 (Thermus sp. (TAP)); AF047381 (Pseudomonas aeruginosa

(phoA)); U49060 (Bacillus subtilis (phoD)); J03930 (Human intestinal (ALPI)); J03252

(Human alkaline (ALPP)); U19108 (Gallus tissue-nonspecific); M13345 (*E. coli*); U31569

(Felis catus (alpl)); L36230 (Zymomonas mobilis (phoD)); M19159 (Human placental heat-

stable (PLAP-1)); M12551 (Human placental (PLAP)); M31008 (Human intestinal); J04948

15   (Human (ALP-1); J03572 (Rat); M61705 (Mouse intestinal (IAP); M61704 (Mouse

embryonic); M61706 (Mouse (AP) pseudogene); M21134 (S.cerevisiae (rALPase)); L07733

(Cow intestinal (IAP)); M18443 (Bovine); M77507 (Synechococcus sp. atypical); M33965

(S.marcescens (phoA)); M33966 (E.fergusonii (phoA)); M29670 (E.coli (phoA)); M29669

(E.coli (phoA)); M29668 (E.coli (phoA)); M29667 (E.coli (phoA)); M29666 (E.coli (phoA));

20   M29665 (E.coli (phoA)); M29664 (E.coli (phoA)); M29663 (E.coli (phoA)); M23549 (Bacillus

subtilis (phoP gene, 3' end and phoR gene); M16775 (B.subtilis phoP); M33634 (B.subtilis

(phoAIII); L27993 (Neurospora crassa); U02550 (Bacillus subtilis (phoA)).

            4)   Luciferase

Any luciferase can be used in the present system. Numerous luciferases are available

25   and have been cloned. For example, the luciferases encoded by nucleic acids with the following

GenBank accession Nos. can be used: AH007711 (Streptomyces clavuligerus (cvm5));

AF124929 (cvm5); U43958 (Cloning vector pRcCMV-luc luciferase gene); M90092

(Xenorhabdus luminescens (luxA)); AF093688 (MMTV-luciferase reporter vector pHH Luc

*SA *PS); AF093687 (MMTV-luciferase reporter vector PHH Luc *SA); AF093686 (MMTV-

30   luciferase reporter vector pHH Luc); AF093685 (Luciferase reporter vector pXP2 *SA *PS);

AF093684 (Luciferase reporter vector pXP2 *SA); AF093683 (Luciferase reporter vector

pXP1); AF093682 (Luciferase reporter vector pXP2); U40374 (Luciferase reporter gene shuttle
vector pMH30); AF003893 (Gonyaulax polyedra luciferase); L39928 (Pyrocoelia miyako
(clone pB-PmL41); L39929 (Hotaria parvula (clone pB-Hp); AF085332 (Gonyaulax polyedra);
U89490 (Vargula hilgendorfii); AF027129 (Eukaryotic luciferase expression vector

5   pCMVtkLUC+); AF027128 (Eukaryotic luciferase expression vector ptkLUC+); AF027127
(Eukaryotic luciferase expression vector pTATALUC+); AF027126 (Eukaryotic luciferase
expression vector pLUC+); U31240 (Photuris pennsylvanica); D25416 (Firefly clone pPFL7);
D25415 (Firefly clone pPFL19); U84006 (Expression vector pBSII-LUCINT firefly luciferase
(LUCINT); U55819 (Plasmid pRL765 with transposon Tn5 and luciferase (luxA and luxB)

10   genes); U55385 (Plasmid pRL1063a with transposon Tn5 and luciferase (luxA and luxB)
genes); U51019 (Luciola lateralis); U49182 (Luciola lateralis); U49181 (Luciola lateralis);
M36597 (K. alfredi symbiont); U47298 (Cloning vector pGL-3-Promoter firefly luciferase
(luc+) gene); U47297 (Cloning vector pGL3-Enhancer firefly luciferase (luc+) gene); U47296
(Cloning vector pGL3-Control firefly luciferase (luc+) gene); U47295 (Cloning vector pGL3-

15   Basic firefly luciferase (luc+) gene); U47123 (Cloning vector pSP-luc+NF, luciferase cassette
fusion vector); U47122 (Cloning vector pSP-luc+, Luciferase cassette vector); M10961
(V.harveyi (luxA and luxB); M65067 (Photobacterium phosphoreum (luxA and luxB); M62917
(Xenorhabdus luminescens (luxA, luxB, luxC, and luxD); M25666 (V.hilgendorfii); M63501
(Renilla reniformis); M15077 (P.pyralis (firefly)); M26194 (Luciola cruciata); M55977

20   (X.luminescens (luxA and luxB)); M90093 (Xenorhabdus luminescens (luxA) and (luxB)
(luxE)); U03687 (Photinus pyralis modified luciferase gene).

5)   Glutathione S-transferase

A glutathione S-transferase (GST), more preferably a *Schistosoma japonicum*
glutathione S-transferase, can be included in the conjugate. GST occurs naturally as a 26 kDa

25   protein which can be expressed in *E. coli* with full enzymatic activity. Conjugates that contain
the full length GST also demonstrate GST enzymatic activity and can undergo dimerization as
observed in nature (Parker, *et al., J. Mol. Biol.*, 213:221 (1990); Ji, *et al., Biochemistry*,
31:10169 (1992); and Maru, *et al., J. Biol. Chem.*, 271:15353 (1996)). The crystal structure of
recombinant *Schistosoma japonicum* GST from pGEX vectors has been determined (McTigue,

30   *et al., J. Mol. Biol.*, 246:21 (1995)) and matches that of the native protein. Conjugates that
contain a GST can be readily purified.

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For example, fusion proteins are easily purified from bacterial lysates by affinity chromatography using Glutathione Sepharose 4B contained in the GST Purification Modules (Amersham Pharmacia Biotech, Inc.). Cleavage of the desired protein from GST is achieved using a site-specific protease whose recognition sequence is located immediately upstream

5   from the multiple cloning site on the pGEX plasmids. Fusion proteins can be detected using a colorimetric assay or immunoassay provided in the GST Detection Module, or by Western blotting with anti-GST antibody. The system has been used successfully in many applications such as molecular immunology (Toye, et al., Infect. Immun., 58:3909 (1990)), the production of vaccines (Fikrig, et al., Science, 250:553 (1990); and Johnson, et al., Nature, 338:585 (1989))

10 and studies involving protein-protein (Kaelin, et al., Cell, 64:521 (1991)) and DNA-protein (Kaelin, et al., Cell, 65:1073 (1991)) interactions.

Any glutathione S-transferase is contemplated. For example, the glutathione S-transferase encoded by nucleic acid with the following GenBank accession Nos. can be used: [AF112567], Fasciola gigantica; [M77682], Fasciola hepatica; [AB016426], Cavia porcellus;

15 [AF144382], Arabidopsis thaliana; [AF133251], Gallus; [AB021655], Issatchenkia orientalis; [AF133268], Manduca sexta; [AF125273], Homo sapiens tissue-type skeletal muscle; [AF125271], Homo sapiens tissue-type pancreas; [AB026292], Sphingomonas paucimobilis; [AB026119], Oncorhynchus nerka; [U49179], Bos taurus; [AF106661], Rattus norvegicus (GstYb4); [L15387], Gallus class-alpha; [AF051318], Clonorchis sinensis; [AF101269],

20 Echinococcus granulosus; [AF077609], Boophilus microplus; [AA956087], Homo sapiens microsomal; [AF004358], Aegilops squarrosa; [AF109714], Triticum aestivum; [U86635], Rattus norvegicus glutathione; [AF111428], Drosophila melanogaster microsomal; [AF111426], Drosophila melanogaster microsomal; [AF071163], Anopheles gambiae; [AF071162], Anopheles gambiae; [AF071161], Anopheles gambiae; [AF071160], Anopheles

25 gambiae; [D10524], Nicotiana tabacum; [AF062403], Oryza sativa; [U77604], Homo sapiens microsomal (MGST2); [U30897], Human (P1b); [U62589], Human (GSTp1c); [U42463], Coccomyxa sp. PA; [AF001779], Sphingomonas paucimobilis strain epa505; [U51165], Cycloclasticus oligotrophus (XYLK); [AF025887], Homo sapiens (GSTA4); [U66342], Plutella xylostella; [AF051238], Picea mariana (Sb52); [AF051214], Picea mariana (Sb18);

30 [AF079511], Mesembryanthemum crystallinum clone R6-R37; [D10026], Rattus norvegicus Yrs-Yrs; [AF048978], Glycine max 2,4-D inducible (GSTa); [AF043105], Homo sapiens (GSTM3); [AF057172], Homo sapiens (GSTT2P); [U21689], Human; [AH006027], Homo sapiens (GSTT2); [AF057176], Homo sapiens (GSTT2); [AF050102], Oryza sativa (GST1);

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[AF044411], Schistosoma japonicum; [U87958], Culicoides variipennis (CVGST1);
[AF026977], Homo sapiens microsomal (MGST3); [AF027740], Homo sapiens microsomal
(MGST1L1); [AF005928], Echinococcus granulosus; [AF001103], Pseudomonas (phnC);
[AF010241], Caenorhabditis elegans (CeGST3); [AF010240], Caenorhabditis elegans
5   (CeGST2); [AF010239], Caenorhabditis elegans (CeGST1); [AF002692], Solanum
commersonii (GST1); [L38503], Homo sapiens (GSTT2); [M97937], E. coli/S. japonicium;
[L29427], Rat GST-P gene; [M14654], Schistosoma japonicum Sj26 antigen; [AB000884], Sus
scrofa; [D44465], Arabidopsis thaliana; [D17673], Arabidopsis thaliana; [D17672],
Arabidopsis thaliana; [U78784], Anopheles dirus; [U71213], Human microsomal; [U70672],
10   Arabidopsis thaliana; [U24428], Mus musculus; [U43126], Naegleria fowleri; [X14233],
D.melanogaster (GST); [L32092], Manduca sexta; [L32091], Manduca sexta; [U30489],
Arabidopsis thaliana; [M24889], Artificial maize; [L05915], Dianthus caryophyllus; [M15872],
Human; [L23766], Oryctolagus cuniculus; [J03679], Solanum tuberosum; [U12472], Human
(GST phi); [U15654], Mus musculus; [M24485], Homo sapiens (GSTP1); [L28771],
15   Onchocerca volvulus; [M14777], Human; [M16594], Human; [M21758], Human; [J03914],
Rat; [K01932], Rat liver; [J02810], Rat prostate; [M25891], Rat; [M11719], Rat liver;
[M28241], Rat; [J03752], Rat; [M73483], Mouse (GST Yc); [J04696], Mouse (GST5-5);
[J04632], Mouse (GST1-1); [M59772], M.auratus; [L20466], Chinese hamster; [M25627],
Human liver; [J03746], Human (SEQ ID No. 137); [M16901], Maize; [M64268], Dianthus
20   caryophyllus; [L11601], Arabidopsis thaliana; [L07589], Arabidopsis thaliana; [M74529],
Oryctolagus cuniculus; [M74528], Oryctolagus cuniculus; [M98271], Schistosoma mansoni 28
kDa; [L23126], Lucilia cuprina; [M95198], Drosophila melanogaster; [L26544], Methylophilus
sp.; [U14753], Dirofilaria immitus; [U12679], Zea mays; [L02321], Human (GSTM5);
[L15386], Chicken.

25   In addition, commercially available Glutathione S-transferase (GST) gene fusion system
can be used. For example, the Glutathione S-transferase (GST) Gene Fusion System
(Amersham Pharmacia Biotech, Inc.) can be used. The system from Amersham Pharmacia
Biotech, Inc. is an integrated system for the expression, purification and detection of fusion
proteins produced in E. coli. The system includes three primary components: pGEX plasmid
30   vectors, various options for GST purification and a variety of GST detection products. A series
of site-specific proteases complements the system. The pGEX plasmids are designed for
inducible, high-level intracellular expression of genes or gene fragments as fusions with
Schistosoma japonicum GST (Smith and Johnson, Gene, 67:31 (1988)). All pGEX Vectors

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(GST Gene fusion) offer: 1) A tac promoter for chemically inducible, high-level expression; 2) an internal *lac I^q* gene for use in any *E. coli* host; 3) very mild elution conditions for release of fusion proteins form the affinity matrix, thus minimizing effects on antigenicity and functional activity; and 4) PreScission, thrombin or factor Xa protease recognition sites for cleaving the desired protein from the fusion product.

The GST Detection Module from Amersham Pharmacia Biotech, Inc. can be used for identification of GST fusion proteins using either a biochemical or immunological assay. In the biochemical assay, glutathione and 1-chloro-2-4-dinitrobenzene (CDNB) serve as substrates for GST to yield a yellow product detectable at 340 nm (Habig, *et al.*, *J. Biol. Chem.*, 249:7130 (1974)). An affinity-purified goat anti-GST polyclonal antibody suitable for Western blots is used in the immunoassay.

The GST 96-Well Detection Module from Amersham Pharmacia Biotech, Inc. contains five microtitre strip plates, horseradish perioxidase (HRP) conjugated anti-GST antibody and recombinant GST protein. The wells of each plate are coated with purified anti-GST antibody to capture GST fusion proteins and are preblocked to provide a low background. HRP conjugated antibody enables sensitive detection of GST proteins.

The anti-GST antibody supplied in the system from Amersham Pharmacia Biotech, Inc. is a polyclonal antibody purified from the sera of goats immunized with purified *schistosomal* glutathione S-transferase (GST). Because of its polyclonal nature, it can recognize more than one epitope on GST, thereby improving its capacity for recognizing GST fusion proteins even if some binding sites are masked due to recombinant protein folding.

Factor Xa can be used for site-specific separation of the GST affinity tag from proteins expressed using pGEX X vectors. Factor Xa enables the site-specific cleavage of fusion proteins containing an accessible Factor Xa recognition sequence. It can be used either following affinity purification or while fusion proteins are bound to Glutathione Sepharose 4B. Factor Xa, purified from bovine plasma, is used to digest fusion proteins prepared from pGEX vectors containing the recognition sequence for factor Xa (pGEX-3X, pGEX-5X-1, pGEX-5X-2 and pGEX-5X-3). It specifically cleaves following the tetrapeptide Ile-Glu-Gly-Arg (SEQ ID No. 139) (Nagai and Thøgersen, *Nature*, 309:810 (1984); and Nagai and Thøgersen, *Methods Enzymol.*, 153:461 (1987)). In the system from Amersham Pharmacia Biotech, Inc., one unit of Factor Xa cleaves ≥ 90% of 100 μg of a test GST fusion protein when incubated in 1 mM CaCl$_2$, 100 mM NaCl and 50 mM Tris-HCl (pH 8.0) at 22°C for 16 hours.

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PreScission protease can be used for site-specific separation of the GST affinity tag
from proteins expressed using pGEX-6P vectors. It enables the low-temperature cleavage of
fusion proteins containing the PreScission Protease recognition sequence. It can be used either
following affinity purification or while fusion proteins are bound to Glutathione Sepharose 4B.

5   PreScission Protease is a genetically engineered fusion protein containing human rhinovirus 3C
protease and GST (Walker, *et al.*, *Bio/Technology*, 12:601 (1994)). This protease was
specifically designed to facilitate removal of the protease by allowing simultaneous protease
immobilization and cleavage of GST fusion proteins produced from pGEX-6P vectors (pGEX-
6P-1, pGEX-6P-2, and pGEX-6P-3). PreScission Protease specifically cleaves between the Gln

10   and Gly residues of the recognition sequence of LeuGluValLeuPheGln/GlyPro (SEQ ID
No. 140) (Cordingley, *et al.*, *J. Bio. Chem.*, 265:9062 (1990)). In the system from Amersham
Pharmacia Biotech, Inc., one unit of PreScission protease will cleave $\geq$ 90% of 100 µg of a test
GST-fusion protein in 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 7.0 at
5°C for 16 hours.

15   Thrombin can be used for site-specific separation of the GST affinity tag from proteins
expressed using pGEX T vectors. It enables the site-specific cleavage of fusion proteins
containing an accessible thrombin recognition sequence. It is purified from bovine plasma;
functionally free of other clotting factors, plasminogen and plasmin. It can be used either
following affinity purification or while fusion proteins are bound to Glutathione Sepharose 4B:

20   Thrombin is used to digest fusion proteins prepared from pGEX vectors containing the
recognition sequence for thrombin (pGEX-1λT, pGEX-2T, pGEX-2TK, pGEX-4T-1, pGEX-
4T2 and pGEX-4T-3). In the system from Amersham Pharmacia Biotech, Inc., one unit of
Thrombin cleaves $\geq$ 90% of 100 µg of a test GST fusion protein when incubated in 1x PBS at
22°C for 16 hours.

25   6)   **Defense proteins**

The conjugates can contain defense protein, such as an antibody. Any antibody,
including polyclonal, monoclonal, single chain or Fab fragments, can be used.

                            7)   **Fluorescent moieties**

The conjugates can contain a fluorescent moiety, such as a green, a blue or a red

30   fluorescent protein. Any green, blue or red fluorescent protein can be used in the present
system. For instance, the green fluorescent proteins encoded by nucleic acids with the

following GenBank accession Nos. can be used: U47949 (AGP1); U43284; AF007834
(GFPuv); U89686 (Saccharomyces cerevisiae synthetic green fluorescent protein
(cox3::GFPm-3) gene); U89685 (Saccharomyces cerevisiae synthetic green fluorescent protein
(cox3::GFPm) gene); U87974 (Synthetic construct modified green fluorescent protein GFP5-

5   ER (mgfp5-ER)); U87973 (Synthetic construct modified green fluorescent protein GFP5
(mgfp5)); U87625 (Synthetic construct modified green fluorescent protein GFP-ER (mfgp4-
ER)); U87624 (Synthetic construct green fluorescent protein (mgfp4) mRNA)); U73901
(Aequorea victoria mutant 3); U50963 (Synthetic); U70495 (soluble-modified green
fluorescent protein (smGFP)); U57609 (enhanced green fluorescent protein gene); U57608

10   (enhanced green fluorescent protein gene); U57607 (enhanced green fluorescent protein gene);
U57606 (enhanced green fluorescent protein gene); U55763 (enhanced green fluorescent
protein (egfp); U55762 (enhanced green fluorescent protein (egfp); U55761 (enhanced green
fluorescent protein (egfp); U54830 (Synthetic *E. coli* Tn3-derived transposon green fluorescent
protein (GF); U36202; U36201; U19282; U19279; U19277; U19276; U19281; U19280;

15   U19278; L29345 (Aequorea victoria); M62654 (Aequorea victoria); M62653 (Aequorea
victoria); AAB47853 ((U87625) synthetic construct modified green fluorescent protein (GFP-
ER)); AAB47852 ((U87624) synthetic construct green fluorescent protein).

Similarly, the blue fluorescent proteins encoded by nucleic acids with the following
GenBank accession Nos. can be used: U70497 (soluble-modified blue fluorescent protein

20   (smBFP); 1BFP (blue variant of green fluorescent protein); AAB16959 (soluble-modified blue
fluorescent protein).

Also similarly, the red fluorescent proteins encoded by nucleic acids with the following
GenBank accession Nos. can be used: U70496 (soluble-modified red-shifted green fluorescent
protein (smRSGFP); AAB16958 ((U70496) soluble-modified red-shifted green fluorescent

25   protein).

2.   Selection of Mutant analyte-binding enzymes

Any mutant analyte-binding enzyme described herein can be used in the conjugate,
including any described herein. In a preferred embodiment, the mutant analyte-binding enzyme
is a mutant SAH hydrolase that at least substantially retains its binding affinity for Hcy or

30   SAH, but has attenuated catalytic activity. Exemplary mutant SAH hydrolases, such as those
set forth above can be included in the conjugate.

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3. Nucleic acids, plasmids and cells

Isolated nucleic acid fragments encoding fusion proteins are provided. The nucleic acid fragment that encodes the fusion protein includes: a) nucleic acid encoding a mutant analyte-binding enzyme, wherein the mutant enzyme has binding affinity for the analyte or an

5   immediate analyte enzymatic conversion product but has attenuated catalytic activity; and b) nucleic acid encoding a protein, peptide or effective fragment thereof that facilitates: i) affinity isolation or purification of the fusion protein; ii) attachment of the fusion protein to a surface; or iii) detection of the fusion protein. Preferably, the nucleic acid is DNA.

Plasmids for replication and vectors for expression that contain the nucleic acid

10   fragments are also provided. Cells containing the plasmids and vectors are also provided. The cells can be any suitable host including, but are not limited to, bacterial cells, yeast cells, fungal cells, plant cells, insect cells and animal cells. The nucleic acids, plasmids, and cells containing the plasmids can be prepared according to methods known in the art including any described herein.

15   In a specific embodiment, a method for producing the above fusion proteins is provided, which method comprises growing cells containing a plasmid encoding the fusion protein under conditions whereby the fusion protein is expressed by the cell, and recovering the expressed fusion protein. Methods for expressing and recovering recombinant proteins are well known in the art (See generally, *Current Protocols in Molecular Biology* (1998) § 16, John Wiley &

20   Sons, Inc.) and such methods can be used for expressing and recovering the expressed fusion proteins. Preferably, the recombinant expression and recovery methods disclosed in Section B.2. can be used.

The recovered fusion proteins can be isolated or purified by methods known in the art such as centrifugation, filtration, chromatograph, electrophoresis, immunoprecipitation, etc., or

25   by a combination thereof (See generally, *Current Protocols in Molecular Biology* (1998) § 10, John Wiley & Sons, Inc.). Preferably, the recovered fusion protein is isolated or purified through affinity binding between the protein or peptide fragment of the fusion protein and an affinity binding moiety. As discussed in the above sections regarding the construction of the fusion proteins, any affinity binding pairs can be constructed and used in the isolation or

30   purification of the fusion proteins. For example, the affinity binding pairs can be protein binding sequences/protein, DNA binding sequences/DNA sequences, RNA binding

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sequences/RNA sequences, lipid binding sequences/lipid, polysaccharide binding sequences/polysaccharide, or metal binding sequences/metal.

4.   Immobilization and supports or substrates therefor

In certain embodiments, where the facilitating agents are designed for linkage to surfaces, recovered, isolated or purified conjugates, such as fusion proteins can be attached to a surface of a matrix material.   Immobilization may be effected directly or via a linker. The conjugates may be immobilized on any suitable support, including, but are not limited to, silicon chips, and other supports described herein and known to those of skill in the art. A plurality of conjugates, which may contain the same or different or a variety of mutant analyte binding enzymes (substrate trapping enzymes) may be attached to a support, such as an array (i.e., a pattern of two, typically three or more) of conjugates on the surface of a silicon chip or other chip for use in high throughput protocols and formats.

It is also noted that the mutant analyte binding enzymes can be linked directly to the surface or via a linker without a facilitating agent linked thereto. Hence chips containing arrays of mutant analyte binding enzymes are contemplated.

For example, an isolated or purified fusion protein can be attached to the surface of a solid or insoluble support, such as a silicon chip, as the intact fusion proteins. Alternatively, the protein or peptide fragment portion can be cleaved off and the mutant analyte-binding enzyme be attached to the surface. The fusion protein can be cleaved by any methods known in the art such as chemical or enzymatic means. The cleavage means must be compatible with the linking sequence between the protein or peptide fragment portion and the mutant analyte-binding enzyme so that the cleavage is linker sequence specific and the cleaved mutant enzyme is functional, i.e., can be used as a substrate-trapping enzyme. Those skilled in the art can readily determine, if necessary, with empirical studies, which cleavage/linker sequence pair to be used. Many cleavage/linker sequence pairs are well known in the art. For example, Factor Xa can be used for site-specific separation of the GST affinity tag from proteins expressed using pGEX X vectors; PreScission protease can be used for site-specific separation of the GST affinity tag from proteins expressed using pGEX-6P vectors; and Thrombin can be used for site-specific separation of the GST affinity tag from proteins expressed using pGEX T vectors.

The matrix material substrates contemplated herein are generally insoluble materials used to immobilize ligands and other molecules, and are those that are used in many chemical syntheses and separations. Such substrates, also called matrices, are used, for example, in

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affinity chromatography, in the immobilization of biologically active materials, and during

chemical syntheses of biomolecules, including proteins, amino acids and other organic

molecules and polymers. The preparation of and use of matrices is well known to those of skill

in this art; there are many such materials and preparations thereof known. For example,

5   naturally-occurring matrix materials, such as agarose and cellulose, may be isolated from their

respective sources, and processed according to known protocols, and synthetic materials may

be prepared in accord with known protocols.

        The substrate matrices are typically insoluble materials that are solid, porous,

deformable, or hard, and have any required structure and geometry, including, but not limited

10   to: beads, pellets, disks, capillaries, hollow fibers, needles, solid fibers, random shapes, thin

films and membranes. Thus, the item may be fabricated from the matrix material or combined

with it, such as by coating all or part of the surface or impregnating particles.

        Typically, when the matrix is particulate, the particles are at least about 10-2000 μM,

but may be smaller or larger, depending upon the selected application. Selection of the

15   matrices will be governed, at least in part, by their physical and chemical properties, such as

solubility, functional groups, mechanical stability, surface area swelling propensity,

hydrophobic or hydrophilic properties and intended use.

        If necessary, the support matrix material can be treated to contain an appropriate

reactive moiety. In some cases, the support matrix material already containing the reactive

20   moiety may be obtained commercially. The support matrix material containing the reactive

moiety may thereby serve as the matrix support upon which molecules are linked. Materials

containing reactive surface moieties such as amino silane linkages, hydroxyl linkages or

carboxysilane linkages may be produced by well established surface chemistry techniques

involving silanization reactions, or the like. Examples of these materials are those having

25   surface silicon oxide moieties, covalently linked to gamma-aminopropylsilane, and other

organic moieties; N-[3-(triethyoxysilyl) propyl]phthelamic acid; and bis-(2-hydroxyethyl)

aminopropyltriethoxysilane. Exemplary of readily available materials containing amino group

reactive functionalities, include, but are not limited to, para-aminophenyltriethyoxysilane.

Silicon or silicon-coated chips and wafers used in high throughput protocols are among those

30   preferred.

        Also derivatized polystyrenes and other such polymers are well known and readily

available to those of skill in this art (e.g., the Tentagel® Resins are available with a multitude of

functional groups, and are sold by Rapp Polymere, Tubingen, Germany; see, U.S. Patent

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No. 4,908,405 and U.S. Patent No. 5,292,814; see, also Butz, *et al.*, *Peptide Res.*, 7:20-23 (1994); and Kleine, *et al.*, *Immunobiol.*, 190:53-66 (1994)).

These matrix materials include any material that can act as a support matrix for attachment of the molecules of interest. Such materials are known to those of skill in this art,

5 and include those that are used as a support matrix. These materials include, but are not limited to, inorganics, natural polymers, and synthetic polymers, including, but are not limited to: cellulose, cellulose derivatives, acrylic resins, glass, silica gels, polystyrene, gelatin, polyvinyl pyrrolidone, co-polymers of vinyl and acrylamide, polystyrene cross-linked with divinylbenzene and others (see, Merrifield, *Biochemistry*, 3:1385-1390 (1964)),

10 polyacrylamides, latex gels, polystyrene, dextran, polyacrylamides, rubber, silicon, plastics, nitrocellulose, celluloses, natural sponges. Of particular interest herein, are highly porous glasses (see, *e.g.*, U.S. Patent No. 4,244,721) and others prepared by mixing a borosilicate, alcohol and water.

Synthetic matrices include, but are not limited to: acrylamides, dextran-derivatives and

15 dextran co-polymers, agarose-polyacrylamide blends, other polymers and co-polymers with various functional groups, methacrylate derivatives and co-polymers, polystyrene and polystyrene copolymers (see, *e.g.*, Merrifield, *Biochemistry*, 3:1385-1390 (1964); Berg, *et al.*, in *Innovation Perspect. Solid Phase Synth. Collect. Pap.*, Int. Symp., 1st, Epton, Roger (Ed), pp. 453-459 (1990); Berg, *et al.*, *Pept., Proc. Eur. Pept. Symp.*, 20th, Jung, G., *et al.* (Eds), pp.

20 196-198 (1989); Berg, *et al.*, *J. Am. Chem. Soc.*, 111:8024-8026 (1989); Kent, *et al.*, *Isr. J. Chem.*, 17:243-247 (1979); Kent, *et al.*, *J. Org. Chem.*, 43:2845-2852 (1978); Mitchell, *et al.*, *Tetrahedron Lett.*, 42:3795-3798 (1976); U.S. Patent No. 4,507,230; U.S. Patent No. 4,006,117; and U.S. Patent No. 5,389,449). Methods for preparation of such matrices are well-known to those of skill in this art.

25 Synthetic matrices include those made from polymers and co-polymers such as polyvinylalcohols, acrylates and acrylic acids such as polyethylene-co-acrylic acid, polyethylene-co-methacrylic acid, polyethylene-co-ethylacrylate, polyethylene-co-methyl acrylate, polypropylene-co-acrylic acid, polypropylene-co-methyl-acrylic acid, polypropylene-co-ethylacrylate, polypropylene-co-methyl acrylate, polyethylene-co-vinyl acetate,

30 polypropylene-co-vinyl acetate, and those containing acid anhydride groups such as polyethylene-co-maleic anhydride, polypropylene-co-maleic anhydride and the like. Liposomes have also been used as solid supports for affinity purifications (Powell, *et al. Biotechnol. Bioeng.*, 33:173 (1989)).

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For example, U.S. Patent No. 5,403,750, describes the preparation of polyurethane-based polymers. U.S. Pat. No. 4,241,537 describes a plant growth medium containing a hydrophilic polyurethane gel composition prepared from chain-extended polyols; random copolymerization is preferred with up to 50% propylene oxide units so that the prepolymer will

5   be a liquid at room temperature. U.S. Pat. No. 3,939,123 describes lightly crosslinked polyurethane polymers of isocyanate terminated prepolymers containing poly(ethyleneoxy) glycols with up to 35% of a poly(propyleneoxy) glycol or a poly(butyleneoxy) glycol. In producing these polymers, an organic polyamine is used as a crosslinking agent. Other matrices and preparation thereof are described in U.S. Patent Nos. 4,177,038, 4,175,183,

10   4,439,585, 4,485,227, 4,569,981, 5,092,992, 5,334,640, 5,328,603.

U.S. Patent No. 4,162,355 describes a polymer suitable for use in affinity chromatography, which is a polymer of an aminimide and a vinyl compound having at least one pendant halo-methyl group. An amine ligand, which affords sites for binding in affinity chromatography is coupled to the polymer by reaction with a portion of the pendant halo-

15   methyl groups and the remainder of the pendant halo-methyl groups are reacted with an amine containing a pendant hydrophilic group. A method of coating a substrate with this polymer is also described. An exemplary aminimide is 1,1-dimethyl-1-(2-hydroxyoctyl)amine methacrylimide and vinyl compound is a chloromethyl styrene.

U.S. Patent No. 4,171,412 describes specific matrices based on hydrophilic polymeric

20   gels, preferably of a macroporous character, which carry covalently bonded D-amino acids or peptides that contain D-amino acid units. The basic support is prepared by copolymerization of hydroxyalkyl esters or hydroxyalkylamides of acrylic and methacrylic acid with crosslinking acrylate or methacrylate comonomers are modified by the reaction with diamines, aminoacids or dicarboxylic acids and the resulting carboxyterminal or aminoterminal groups are condensed

25   with D-analogs of aminoacids or peptides. The peptide containing D-aminoacids also can be synthesized stepwise on the surface of the carrier. For example, U.S. Patent No. 4,178,439 describes a cationic ion exchanger and a method for preparation thereof. U.S. Patent No. 4,180,524 describes chemical syntheses on a silica support.

The fusion protein can be attached to the surface of the matrix material by methods

30   known in the art. Numerous methods have been developed for the immobilization of proteins and other biomolecules onto solid or liquid supports (see, e.g., Mosbach, *Methods in Enzymology*, 44 (1976); Weetall, *Immobilized Enzymes, Antigens, Antibodies, and Peptides*, (1975); Kennedy, *et al.*, *Solid Phase Biochemistry, Analytical and Synthetic Aspects*, Scouten,

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ed., pp. 253-391 (1983); see, generally, Affinity Techniques. Enzyme Purification: *Part B. Methods in Enzymology*, Vol. 34, ed. W. B. Jakoby, M. Wilchek, Acad. Press, N.Y. (1974); and Immobilized Biochemicals and Affinity Chromatography, *Advances in Experimental Medicine and Biology*, vol. 42, ed. R. Dunlap, Plenum Press, N.Y. (1974)).

5   Among the most commonly used methods are absorption and adsorption or covalent binding to the support, either directly or via a linker, such as the numerous disulfide linkages, thioether bonds, hindered disulfide bonds, and covalent bonds between free reactive groups, such as amine and thiol groups, known to those of skill in art (see, *e.g.*, the PIERCE CATALOG, ImmunoTechnology Catalog & Handbook, 1992-1993, which describes the

10   preparation of and use of such reagents and provides a commercial source for such reagents; Wong, *Chemistry of Protein Conjugation and Cross Linking*, CRC Press (1993); see also DeWitt, *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, 90:6909 (1993); Zuckermann, *et al.*, *J. Am. Chem. Soc.*, 114:10646 (1992); Kurth, *et al.*, *J. Am. Chem. Soc.*, 116:2661 (1994); Ellman, *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, 91:4708 (1994); Sucholeiki, *Tetrahedron Lttrs.*, 35:7307 (1994); Su-

15   Sun Wang, *J. Org. Chem.*, 41:3258 (1976); Padwa, *et al.*, *J. Org. Chem.*, 41:3550 (1971); and Vedejs, *et al.*, *J. Org. Chem.*, 49:575 (1984), which describe photosensitive linkers).

         To effect immobilization, a composition containing the protein or other biomolecule is contacted with a support material such as alumina, carbon, an ion-exchange resin, cellulose, glass or a ceramic. Fluorocarbon polymers have been used as supports to which biomolecules

20   have been attached by adsorption (see, U.S. Patent No. 3,843,443; Published International PCT Application WO/86 03840).

         A large variety of methods are known for attaching biological molecules, including proteins and nucleic acids, molecules to solid supports (see, *e.g.*, U.S. Patent No. 5451683). For example, U.S. Pat. No. 4,681,870 describes a method for introducing free amino or

25   carboxyl groups onto a silica matrix. These groups may subsequently be covalently linked to other groups, such as a protein or other anti-ligand, in the presence of a carbodiimide. Alternatively, a silica matrix may be activated by treatment with a cyanogen halide under alkaline conditions. The anti-ligand is covalently attached to the surface upon addition to the activated surface. Another method involves modification of a polymer surface through the

30   successive application of multiple layers of biotin, avidin and extenders (see, *e.g.*, U.S. Patent No. 4,282,287). Other methods involve photoactivation in which a polypeptide chain is attached to a solid substrate by incorporating a light-sensitive unnatural amino acid group into the polypeptide chain and exposing the product to low-energy ultraviolet light (see, *e.g.*, U.S.

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Patent No. 4,762,881). Oligonucleotides have also been attached using a photochemically active reagent, such as a psoralen compound, and a coupling agent, which attaches the photoreagent to the substrate (see, *e.g.*, U.S. Patent No. 4,542,102 and U.S. Patent No. 4,562,157). Photoactivation of the photoreagent binds a nucleic acid molecule to the

5   substrate to give a surface-bound probe.

Covalent binding of the protein or other biomolecule or organic molecule or biological particle to chemically activated solid matrix supports such as glass, synthetic polymers, and cross-linked polysaccharides is a more frequently used immobilization technique. The molecule or biological particle may be directly linked to the matrix support or linked via linker,

10   such as a metal (see, *e.g.*, U.S. Patent No. 4,179,402; and Smith, *et al.*, *Methods: A Companion to Methods in Enz.*, 4:73-78 (1992)). An example of this method is the cyanogen bromide activation of polysaccharide supports, such as agarose. The use of perfluorocarbon polymer-based supports for enzyme immobilization and affinity chromatography is described in U.S. Pat. No. 4,885,250. In this method the biomolecule is first modified by reaction with a

15   perfluoroalkylating agent such as perfluorooctylpropylisocyanate described in U.S. Pat. No. 4,954,444. Then, the modified protein is adsorbed onto the fluorocarbon support to effect immobilization.

The activation and use of matrices are well known and may be effected by any such known methods (see, *e.g.*, Hermanson, *et al.*, *Immobilized Affinity Ligand Techniques*,

20   Academic Press, Inc., San Diego (1992)). For example, the coupling of the amino acids may be accomplished by techniques familiar to those in the art and provided, for example, in Stewart and Young, *Solid Phase Synthesis*, Second Edition, Pierce Chemical Co., Rockford (1984).

Other suitable methods for linking molecules to solid supports are well known to those

25   of skill in this art (see, *e.g.*, U.S. Patent No. 5,416,193). These include linkers that are suitable for chemically linking molecules, such as proteins, to supports and include, but are not limited to, disulfide bonds, thioether bonds, hindered disulfide bonds, and covalent bonds between free reactive groups, such as amine and thiol groups. These bonds can be produced using heterobifunctional reagents to produce reactive thiol groups on one or both of the moieties and

30   then reacting the thiol groups on one moiety with reactive thiol groups or amine groups to which reactive maleimido groups or thiol groups can be attached on the other.

Other linkers include, acid cleavable linkers, such as bismaleimideothoxy propane, acid labile-transferrin conjugates and adipic acid dihydrazide, that would be cleaved in more acidic

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intracellular compartments; cross linkers that are cleaved upon exposure to UV or visible light and linkers, such as the various domains, such as C_H1, C_H2, and C_H3, from the constant region of human IgG_1 (Batra, *et al.*, *Molecular Immunol.*, 30:379-386 (1993)). Presently preferred linkages are direct linkages effected by adsorbing the molecule to the surface of the matrix.

5   Other linkages are photocleavable linkages that can be activated by exposure to light (see, *e.g.*, Goldmacher, *et al.*, *Bioconj. Chem.*, 3:104-107 (1992)). The photocleavable linker is selected such that the cleaving wavelength does not damage linked moieties. Photocleavable linkers are linkers that are cleaved upon exposure to light (see, *e.g.*, Hazum, *et al.*, *Pept., Proc. Eur. Pept. Symp.*, 16th, Brunfeldt, K (Ed), pp. 105-110 (1981), which describes the use of a

10   nitrobenzyl group as a photocleavable protective group for cysteine; Yen, *et al.*, *Makromol. Chem.*, 190:69-82 (1989), which describes water soluble photocleavable copolymers, including hydroxypropylmethacrylamide copolymer, glycine copolymer, fluorescein copolymer and methylrhodamine copolymer; Goldmacher, *et al.*, *Bioconj. Chem.*, 3:104-107 (1992), which describes a cross-linker and reagent that undergoes photolytic degradation upon exposure to

15   near UV light (350 nm); and Senter, *et al.*, *Photochem. Photobiol.*, 42:231-237 (1985), which describes nitrobenzyloxycarbonyl chloride cross linking reagents that produce photocleavable linkages). The selected linker will depend upon the particular application and, if needed, may be empirically selected.

In a preferred embodiment, the recovered fusion protein is attached to the surface

20   through affinity binding between the protein or peptide fragment of the fusion protein and an affinity binding moiety on the surface.

5.   Use of the conjugates in assays

In a specific embodiment, a method for assaying an analyte in a sample is provided, which method comprises: 1) contacting the sample with a conjugate that contains: a) at least on

25   mutant analyte-binding enzyme, and b) a facilitating agent that, for example, facilitates: i) affinity isolation or purification of the fusion protein; ii) attachment of the conjugate to a surface; or iii) detection of the conjugate; and 2) detecting binding between the analyte or the immediate analyte enzymatic conversion product and the conjugate, whereby, for example, the presence or amount of the analyte in the sample is assessed.

30   In some embodiments, the conjugate is a fusion protein, which prior to the contact between the sample and the fusion protein, is isolated or purified. More preferably, the fusion protein is isolated or purified through affinity binding between the protein or peptide fragment

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of the fusion protein and an affinity binding moiety. Any kind of affinity interaction can be used for isolating or purifying the fusion protein. The affinity interactions, such as those desribed herein, but not limited to, are protein/protein, protein/nucleotide, protein/lipid, protein/polysaccharide, or protein/metal interactions.

5   In other embodiments, prior to the contact between the sample and the conjugate, such as a fusion protein, the conjugate is attached to a surface. More preferably, the conjugate is attached to the surface through affinity binding between the facilitating agent of conjugate and an affinity binding moiety on the surface. Any kind of affinity interaction can be used for attaching the conjugate, including the protein/protein, protein/nucleotide, protein/lipid,

10   protein/polysaccharide, or protein/metal interactions.

          Any analytes, particular small molecule analytes can be assayed using the above assay methods. For example, the analyte to be analyzed is Hcy and the mutant analyte-binding enzyme of the fusion protein is a mutant Hcy-binding enzyme.

          The following examples are included for illustrative purposes only and are not intended

15   to limit the scope of the invention.

EXAMPLE 1

Preparation of mutant SAH hydrolase-encoding nucleic acid

          Human placental SAH hydrolase gene (SEQ ID No. 1) was subcloned into an expression vector pKK223-3 (Pharmacia Biotech, Piscataway, New Jersey) at the EcoR I site.

20   pKK223-3 contains the strong tac promoter upstream from the multiple cloning site and the strong rrnB ribosomal terminator downstream for control of protein expression. The SAH hydrolase gene-containing expression vector was transferred into an *E. coli* strain JM109 (Invitrogen, Carlsbad, CA). Site-directed mutagenesis of SAH hydrolase was conducted in two ways: 1) single-strand DNA-based M13 method; and 2) double-strand DNA-based PCR

25   method.

Single-strand DNA-based mutagenesis

          Single-strand DNA-based mutagenesis was conducted based on the method described by Taylor, *et al.*, *Nucleic Acids Res.*, 13:8765-8785 (1985), which exploits the inability of NciI to cleave a thio-containing DNA strand. Sculptor™ invitro mutagenesis system RPN1526

30   (Amersham Life science, UK) was used. The pKK223-3 vector containing the wild type gene

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of SAH hydrolase was prepared using the method of alkaline lysis followed by plasmid
purification using Promega's DNA purification kit (Wizard plus Minipreps, Promega, Madison
WI). The purified plasmid was digested with EcoR I (Stratagene, La Jolla, CA) at 37°C for 2
hours to obtain the EcoR I fragment by agarose gel electrophoresis followed by DNA

5   purification using Promega DNA purification kit. The purified EcoR I fragment was subcloned
into M13 mp19 DNA (Pharmacia Biotech, Piscataway, New Jersey) by T4 DNA ligase
(Pharmacia Biotech Piscataway, New Jersey). The ligation was conducted in One-phor-All
buffer (10 mM tris-Ac, pH 7.5, 10 mM Mg(Ac)2, 50 mM KAc; Pharmacia LKB Biotechnology
AB, Uppsala, Sweden) at 4°C overnight. The ligation product was transferred into TG1 cells

10   (Stratagene, La Jolla, CA) by incubation of 10 μl of the M13 with 90 μl of competent TG 1
cells at 0°C for 30 min. and 42°C for 75 sec. After being chilled to 0°C for 2 min, 500 μl of
2XYT media was added to the cells and incubated for 10 min. at 37°C. Two hundred μl of
growing nontransformed TG1 cells were mixed with the transformed TG1 cells, and to which
2.5 ml of soft agarose LB (42°C) was added. The cell mixture was immediately poured onto

15   preheated LB agar plates (40°C), and incubated at 37°C overnight. Phage clones were picked
up for examination of the insertion of SAH hydrolase gene and the orientation through DNA
sequencing and restriction enzyme analysis. The selected phage clone was used for preparation
of single strand DNA template.

          The M13 phage containing the SAH hydrolase gene were incubated with TG1 cells in 3

20   ml of 2xYT media overnight. One drop of the overnight culture was mixed with growing TG1
cells (in log phase) in 30 ml of 2XYT media. Cells were incubated for 8 hours with shaking.
After centrifugation, the supernatant was collected for single-strand template DNA purification.
The purification was conducted according to the manufacture's procedure provided by
Amersham Life Science.

25   **Design of primers for point mutation**

          Oligonucleotides (15-30 bases) were synthesized by CruaChem (Sterling, VA). The
sequence of the oligonucleotides were designed to be complementary to the sequence in the
region covering both sides of the mutation site. For example, to mutate lys 426 to glu 426, the
oligonucleotides used as primer contained the following sequence:

30   GGCCCCTTC**GAG**CCGGATCACTACCGC (SEQ ID No. 141) where **GAG** codes for glu
instead of original (wild type) **AAG** which codes for lys.

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The selection of mutation sites was based on x-ray structure of the substrate binding site and coenzyme binding site of human SAH hydrolase (Turner, *et al.*, *Nature Structural Biology*, 5:369-376 (1998)). Amino acid residues such as Thr 157, Asp 131, Hys 301, Lys 186, Asn 191, Glu 156, Asp 190, Phe 362, Phe 302, Asn 181, His 353, Glu 59, Ser 83, His 55,

5   Leu 54, Cys 79, His 301, Arg 343, Asp 303, Leu 344, Asn 80, Asn 346, Asp 107 and entire C-terminal residues can be the mutagenesis targets (see Table 2 for particular mutations generated). The coenzyme binding domain contains residues from Tyr193-Asn346.

The oligonucleotides were dissolved in water to a concentration of 5 ng/μl. The oligonucleotide solution was then phosphorylated at the 5'-end using polynucleotide kinase.

10   The phosphorylation reaction mixture contained the following materials: 2.5 μl of oligonucleotides (5 ng/μl), 3 μl of one-phor-all 10X kinase buffer (Pharmacia Biotech), 21.5 μl of water, 2 μl of 10 mM ATP, and 1 μl of polynucleotide kinase (100,000U/ml) (Pharmacia Biotech). The reaction mixture was incubated at 37°C for 30 min. followed by heating at 70°C for 10 min. to inactivate the enzyme.

15

Table 8.   Oligonucleotides used for site-directed mutagenesis of human SAH hydrolases

Mutant	Mutagenic oligonucleotide	Codon Change	SEQ ID
K186A	GACTTCGTCACC**GCC**AGCAAGTTTGGG	AAG→GCC	142
F302S	AACATTGGACAC**TCT**GACGTGGAGATC	TTT→TCT	143
H301D	TGTAACATTGGA**GAC**TTTGACGTGGAG	CAC→GAC	144
H353S	TGTGCCATGGGC**TCC**CCCAGCTTCGTG	CAC→TCC	145
R343A	CTGGCCGAGGGT**GCG**CTGGTCAACCTG	CGG→GCG	146
D190A	AAGAGCAAGTTT**GCC**AACCTCTATGGC	GAC→GCC	147
F82A	AGCTGCAACATC**GCC**TCCACCCAGGAC	TTC→GCC	148
N181D	AACCTCTATGGC**GAC**CGGGAGTCCCTC	AAT→GAC	149
R431A	CCGGATCACTAC**GCC**TACTGAGAATTC	CGC→GCC	150
K426R	TGTGATGGCTTC**CGC**CCGGATCACTAC	AAG→CGC	151
C195S	AACCTCTATGGC**TCC**CGGGAGTCCCTC	TGC→TCC	152
Δ432	GATCACTACCGC**TGA**TGAGAATTCGAG	ATC→TGA	153

The mutagenized codon is underlined, and the nucleotides changed are in boldface type.

Table 9.   **Additional oligonucleotides used for site-directed mutagenesis of human SAH hydrolases**

Sequence ID	Sequence	Seq. ID No.	F/R
Glu156Ala	GGCATCTCTGAGGCGACCACGACTGGG	155	Fo
Glu156Ala	CCCAGTCGTGGTCGCCTCAGAGATGCC	156	Re
Glu156Asp	GGCATCTCTGAGGACACCACGACTGGG	157	Fo
Glu156Asp	CCCAGTCGTGGTGTCCTCAGAGATGCC	158	Re
Asp131Lys	CTCAACATGATTCTGGACAAGGGGGGCGACCTCACC	159	Fo
Asp131Lys	GGTGAGGTCGCCCCCCTTGTCCAGAATCATGTTGAG	160	Re
Asp131Asn	CTCAACATGATTCTGGACAACGGGGGCGACCTCACC	161	Fo
Asp131Asn	GGTGAGGTCGCCCCCGTTGTCCAGAATCATGTTGAG	162	Re
Lys186Ala	GACTCCGTCACCGCGAGCAAGTTTGAC	163	Fo
Lys186Ala	GTCAAACTTGCTCGCGGTGACGGAGTC	164	Re
Lys186Asp	GACTCCGTCACCGACAGCAAGTTTGAC	165	Fo
Lys186Asp	GTCAAACTTGCTGTCGGTGACGGAGTC	166	Re
His55Pro	GCTGGCTGCCTGCCCATGACCGTGGAGACG	167	Fo
His55Pro	CGTCTCCACGGTCATGGGCAGGCAGCCAGC	168	Re
Arg343Ala	CTGCTGGCCGAGGGTGCGCTGGTCAACCTG	169	Fo
Arg343Ala	CAGGTTGACCAGCGCACCCTCGGCCAGCAG	170	Re
Asp303Glu	GTGTGTAACATTGGACACTTTGAGGTGGAGATCGATGTC	171	Fo
Asp303Glu	GACATCGATCTCCACCTCAAAGTGTCCAATGTTACACAC	172	Re
Phe302Ile	GTGTGTAACATTGGACACATTGACGTGGAGATC	173	Fo
Phe302Ile	GATCTCCACGTCAATGTGTCCAATGTTACACAC	174	Re
Leu344Gly	GCCGAGGGTCGGGGGGTCAACCTGGGTTGTGCC	175	Fo
Leu344Gly	GGCACAACCCAGGTTGACCCCCCGACCCTCGGC	176	Re
Phe82Ser	CAGTGGTCCAGCTGCAACATCTCCTCCACCCAGGAC	177	Fo
Phe82Ser	GTCCTGGGTGGAGGAGATGTTGCAGCTGGACCACTG	178	Re
Thr159Ser	GAGGAGAGGACGTCCGGGGTCCACAACCTC	179	Fo
Thr159Ser	GAGGTTGTGGACCCCGGACGTCCTCTCCTC	180	Re
Asn346Gly	GGTCGGCTGGTCGGCCTGGGTTGTGCC	181	Fo
Asn346Gly	GGCACAACCCAGGCCGACCAGCCGACC	182	Re
Asn346Asp	GGTCGGCTGGTCGACCTGGGTTGTGCC	183	Fo
Asn346Asp	GGCACAACCCAGGTCGACCAGCCGACC	184	Re
Cys79Ala	GTGCAGTGGTCCAGCGCCAACATCTTCTCCACC	185	Ro
Cys79Ala	GGTGGAGAAGATGTTGGCGCTGGACCACTGCAC	186	Re
Cys79Gly	GTGCAGTGGTCCAGCGGCAACATCTTCTCCACC	187	Fo
Cys79Gly	GGTGGAGAAGATGTTGCCGCTGGACCACTGCAC	188	Re
His301Ala	GTGTGTAACATTGGAGCCTTTGACGTGGAG	189	Fo
His301Ala	CTCCACGTCAAAGGCTCCAATGTTACACAC	190	Re
Asp303Ala	GTGTGTAACATTGGACACTTTGCCGTGGAG	191	Fo
Asp303Ala	GACATCGATCTCCACGGCAAAGTGTCCAATGTTACACAC	192	Re

F: forward oligonucleotide
R: backward oligonucleotide.

5

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The 5'-phosphorylated oligonucleotides DNA was annealed with single-stranded DNA (M13 phage containing wild type human SAH hydrolase gene, 1µg/µl) in a ratio of oligonucleotide: template of 2:1 in annealing buffer. The annealing reaction was performed by incubating the annealing mixture at 70°C for 3 min. followed by 30 min. at 37°C or followed by

5   transferring the micro centrifuge tube to a 55°C beaker and then allowed to cool to room temperature. To the annealing mixture (17 µl), 19 µl of dNTP A (α-S) mix, 1.5 µl of T7 DNA polymerase (0.8 units), and 2.5 µl of T4 DNA ligase (92.5 units), and 6 µl of water were added. After 10 min. at room temperature and 30 min. at 37°C, the reaction was stopped by heat inactivation at 70°C for 15 min. To the reaction mixture was added T5 exonuclease (2000

10 units) and exonuclease buffer to remove single-strand non-mutant DNA at 37°C for 30 min. followed by 15 min. of heat inactivation at 70°C. NciI (5 units) was added to the reaction mixture to nicking the non-mutant strand by incubating NciI at 37°C for 90 min. The non-mutant strand was digested by adding 160 units of Exonuclease III and incubating at 37°C for 30 min. followed by heat inactivation. To repolymerize the gaped DNA, dNTP mix B and 3.5

15 units of DNA polymerase I and 2.5 units of T4 DNA ligase were added to the reaction mixture, and incubated at 37°C for 1 h.

The M13 plasmid containing the mutated SAH hydrolase gene was then transferred into competent TG 1 host cells by heat shock method or an electroporation method. Ten µl of the mutant M13 plasmid was added to 90 µl of water and mixed with competent TG1 cells in ice

20 for 40 min. The TG1 cells were shocked by incubation at 42°C for 45 sec. and immediately at 0°C for 5 min. The transferred TG1 cells were allowed to return to room temperature, and mixed with 200 µl of growing non-transferred TG1 cells (served as lawn cells). Three ml of molten Htop agar was added and mixed followed by immediately pouring the cells onto a L plate. The plate was incubated in 37°C for overnight. Phage plaques formed were picked by

25 sterile tooth pick and swirling in a tube containing 3 ml of 2XYT medium. After overnight culture, cells were collected by centrifugation, and the double-strand M13 plasmid from the cells was purified by using Promega DNA purification kit (Wizard plus Minipreps).

The supernatant from centrifugation was used to purify single-strand M13 DNA. The mutation was confirmed by DNA sequencing of the single-strand M13 DNA using Sequenase

30 Version 2.0 (Unites States Biochemical). The double-strand M13 DNA containing correct mutation sequence was selected, and digested with EcoR I. The EcoR I fragment containing

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the mutant SAH hydrolase gene was purified by agarose electrophoresis followed by gene

cleaning using Qlaquick Gel Extraction kit (Qiagen, Valencia, CA). The purified EcoR.I

fragment was subcloned into pKK223-3 expression vector using T4 ligase. Two µl of EcoR 1

treated and 5'-dephosphorylated pKK223-3 vector backbone was incubated with 10 µl of the

5   purified mutant insert DNA in a backbone to insert ratio of 2:1. The ligation reaction was

carried out in One-phore-All buffer containing 0.01 M ATP at 16C overnight. The ligated

vector containing mutant SAH hydrolase gene was transferred into competent *E. Coli* JM109

cells by heat shock method. The transformed cells were selected against 100 µl/ml ampicillin.

Ampicillin-resistant clones were picked and grown in 10 ml of 2xYT medium containing 35

10   µl/ml ampicillin for 2 hours at 37°C and then induced with 1 mM isopropyl-1-thio-β-D-

galactopyranoside (IPTG) and grown overnight at 37°C. The cells were harvested by

centrifugation, and suspended in 0.8 ml of 50 mM Tri-HCl, pH 7.5, containing 2 mM EDTA.

Cells were lysed by rapid freezing and thawing. After centrifugation at 13,500 rpm for 1 hour

at 4°C, the supernatant was collected for SDS-PAGE analysis for over-expression of SAH

15   hydrolase mutant protein. A heavy protein band at molecular size of 47,000 Da indicates the

overexpression of mutant SAH hydrolase protein.

PCR-based mutagenesis method

PCR-based mutagenesis was performed using the ExSite PCR-based Site-Directed

Mutagenesis Kit (Stratagene, La Jolla, CA). The ExSite method uses increased template

20   concentration and <10 PCR cycles. The resulting mixture of template DNA, newly synthesized

DNA and hybrid parental/newly synthesized DNA is treated with Dpn I and Pfu DNA

polymerase. Dpn I digests the *in vivo* methylated parental template and hybrid DNA, and Pfu

DNA polymerase polishes the ends to create a blunt-ended PCR product. The end-polished

PCR product is then intramolecularly ligated together and transformed into *E. coli* cells. The

25   detailed experimental procedure is described as follows:

To a microcentrifuge tube were added 0.5 pmol of template DNA, 2.5 µl of 10x

mutagenesis buffers, 1µl of 25 mM dNTP mix, 15 pmol of each primer, and ddH$_2$O to a final

volume of 24 µl. To the reaction mixture was then added 1 µl of ExSite DNA polymerase

blend (5 U/µl). The reaction solution was overlayed with 20 µl of mineral oil and thermal cycle

30   the DNA using 7012 amplification cycles. The cycling parameters are listed in Table 10.

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Table 10. Mutagenesis Cycling Parameters

Segment	Cycles	Temperature	Time
1	1	94°C	4 min.
		50°C	2 min.
		72°C	2 min.
2	8	94°C	1 min.
		56°C	2 min.
		72°C	1 min.
		72°C	5 min.
3		72°C	5 min.

Following amplification, the reaction tube was placed on ice for 2 min. to cool the

reaction to <37°C. To the reaction tube were added 1 μl of the Dpn I restriction enzyme (10

5   U/μl) and 0.5 μl of cloned Pfu DNA polymerase (2.5 U/μl) followed by incubation at 37°C for

30 min. The reaction was stopped by heating at 72°C for 30 min. For ligating the product, to

the reaction tube were added 100 μl of ddH2O, 10 μl of 10x mutagenesis buffer, and 5 μl of 10

mM rATP. Transfer 10 μl of the above reaction mixture to a new micocentrifuge tube and add

1 μl of T4 DNA ligase (4 U/μl). The ligation was incubated at 37°C for 1 hour. 2 μl of the

10   ligated DNA was added to 80 μl of Epicurian Coli XL1-Blue supercompetent cells on ice and

incubated for 30 min. followed by 45 seconds at 42defendant and 2 min. on ice. The

transformed cells were immediately plated on LB-ampicillin agar plates which had been spread

with 20 μl of 10% X-gal prepared in DMF and 20 μl of 100 M IPTG in H2O. The plate was

incubated overnight at 37°C. The blue colonies were selected as colonies containing the

15   mutagenized plasmid. The selected colonies were further confirmed by DNA sequencing.

Protein overexpression and substrate trapping screening were performed as described above.

       Double-strand pKK223-3 containing human SAH hydrolase (wild type) was purified

from 50 ml of E. coli JM109 culture using Promega DNA purification kit (Wizard plus

Minipreps). The purified plasmid was annealed with PCR primers containing the desired

20   mutation sequence.

       Deletion and insertion mutations were also performed according to the manufacture's

protocol using ExSite PCR-based Site-directed Mutagenesis Kit. Double mutations or

combinations of mutation and deletion or insertion were carried out using mutated or deleted

DNA as template for secondary mutation or deletion using either M13-based mutagenesis or

25   PCR-based mutagenesis methods.

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Identification of substrate trapping SAH hydrolase

The cell-free extracts from colonies that inducibly overexpressed mutant SAH hydrolase proteins were chromatographed on a monoQ column (HR5/5) equipped with FPLC system. Proteins were eluted with a linear gradient of NaCl from 0 to 1 M in 10 mM sodium

5   phosphate buffer, pH 7.0 over 35 min. The major protein peak that eluted at the same or close retention time as that of the wild type SAH hydrolase was collected. An aliquot collected mutant SAH hydrolase (1-10 μg) was incubated with [^{3}H]SAH (10 mCi/mmole, 200 μM) and 30 μM of 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB) at room temperature for 5-30 min.

The reaction solution was filtered through a membrane of molecular weight cut-off at

10   30,000 by centrifugation. The filtrate was measured at 412 nm for Hcy formation (enzyme activity) and the [^{3}H] radioactivity on the membrane was measured by scintillation counting after membrane washing with 1 ml of 50 mM phosphate buffer, pH 7.0.

The mutant hydrolases that show high radioactivity on the membrane and low O.D. at 412 nm of the filtrate relative to the wild type enzyme were selected as candidates for further

15   characterization including determination of Km or Kd and binding energy (ΔG). Mutant SAH hydrolases with Km value lower than 10 μM toward SAH and kcat value lower than 0.1 per second were overexpressed in larger quantity (1-2 L of *E. coli* culture) and the enzyme proteins were purified to homogenous as judged by single band on SDA-PAGE.

EXAMPLE 2

20   Large scale overexpression and purification of wild type and mutant forms of SAH hydrolases

Purification

The cell-free extract of IPTG-induced *E. Coli* JM109 (containing SAH hydrolase gene in pKK223-3 vector) culture was mixed with powder DEAE-cellulose (Sigma, St. Louis, MO) equilibrated with 0.1 M sodium phosphate buffer, pH 7.2 containing 1 mM EDTA (buffer A).

25   The cell-free extract and DEAC-cellulose mixture was placed in a funnel and filtrated under vacuum. After washing with 3 volumes of buffer A, the filtrate was precipitated by solid ammonium sulfate (30-60%). The precipitated protein was collected by centrifugation at 13000 rpm, and resuspended in 50 mM sodium phosphate buffer, pH 7.2, containing 1 mM EDTA. The protein was chromatographed through a Sephacryl S-300 size exclusion column

30   (2.5X100 cm) (Pharmacial Biotech, Piscataway, New Jersey) followed by a DEAE-Sepharose ion exchange column (2.5X30 cm) eluted by a linear NaCl gradient. The major protein peak

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from DEAE-Sepharose was examined by SDS-PAGE. In most of the times, this purification procedure gave a single protein band on SDS-PAGE. Sometime, minor bands were observed on SDS-PAGE. In this case, rechromatography on DEAE-Sepharose column was performed to obtain pure protein. SAH hydrolase activity or [³H]SAH binding affinity was also measured to

5   confirm the protein peak.

Storage of the purified SAH hydrolase

The purified wild type and mutant SAH hydrolases were dialyzed against 5 mM sodium phosphate buffer, pH 7.0 for 6 hours at 4°C. The protein was then frozen in liquid nitrogen and lyophilized under vacuum. The lyophilized protein was stored at -70°C. The protein was stable

10   for at least 2 years. The purified protein can also be stored in liquid containing 20% of glycerol at -20°C. For wild type enzyme, addition of 5 mole excess of adenosine (Ado) to the 20% glycerol solution stabilizes the enzyme activity even better.

Assays for enzyme activity

The assay of SAH hydrolase activity in the hydrolytic direction was performed as

15   described in Yuan, et al., J. Biol. Chem., 271:28008-28016, 1996). The assay measures the hydrolysis of SAH into Ado and Hcy. The reaction product Hcy was derivatized by thiol specific reagent DTNB for colometric determination at 412 nm. The assay for SAH hydrolase in the synthetic direction was measured by the formation of SAH from substrate Ado and Hcy using HPLC (see, Yuan, et al., J. Biol. Chem., 268:17030-17037 (1993). One unit of the

20   enzyme activity was defined as the amount of enzyme that can hydrolyze or synthesize 1 μ mole of SAH/min/mg.

Assay for binding affinity (Kd)

For mutant enzyme that completely lacks activity, the binding constant (Kd) values were determined by an equilibrium dialysis technique using [³H] SAH and Spectrum 5-cell

25   Equilibrium Dialyzer) (Spectrum, Houston, Texas). The membrane disc used had molecular cut-off of 25,000.

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EXAMPLE 3

Preparation of reagents

Preparation of fluorophore-labeled Ado and SAH analogs

Method 1

5   Ado-5'-carboxylic acid (Sigma, St. Louis, MO) was derivatized with 9-

(hydroxylmethyl)anthracene (HMA) (Fluka, Buchs, Switzerland). To 10 mg of Ado-5'-

carboxylic acid dissolved in 100 ml of chloroform (10 min sonication) was added 50 mg 1-

hydroxybenzotriazole (HOBT) (Janssen Chimica, Beerse, Belgium). After evaporation to

dryness under nitrogen, 300 mg of N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide

10   hydrochloride in 300 ml chloroform and 5 ml of triethylamine were added. The resulting

solution was kept at 0°C for 30 min. To the above reaction mixture was added 200 mg HMA in

100 ml of chloroform. The mixture was allowed to stand at room temperature for 10 min. and

then evaporated to dryness under a stream of nitrogen. The residue obtained was dissolved in

10 ml of HPLC mobile phase (methanol-water mixture, 90:10, w/w). One ml of the above

15   solution was injected into a semi-preparative column (Econosphere, C18, 7x300 mm, Alltech,

Dearfield, IL). The column was eluted with an isocratic method. The flow rate was 2 ml/min.

The peaks were monitored at UV260 nm and fluorescence at Ex-365nm, Em-415nm. The

peaks with UV and fluorescence absorbance were collected as HMA-labeled Ado-5'-ester.

Method 2

20   Ado-5'caroboxylic acid and 4-bromomethyl-7-methoxycoumarin (Br-Mmc) (Sigma, St.

Louis, MO) were dissolved in ethyl acetate in a molar ratio of 1:3. The reaction volume was 25

ml. After addition of 2 g of finely powdered K_2CO_3 the solution was refluxed for 1 hour using

a ml-reluxer. After cooling, the reaction solution was injected into a C18 column

(Econosphere, C18, 7x300 mm, Alltech, Deerfield, IL) for HPLC separation. The elution was

25   monitored by UV (260 nm) and fluorescence (Em 328nm and Ex390nm). The elution was

performed in a linear gradient of methanol:water from 20 to 100% over 30 min. The flow rate

was 2 ml/min.

Method 3

This method is depicted in Figure 3. Adenosyl-L-cysteine (Ado-Cys) and 4-

30   Bromomethyl-7-methoxycoumarin (Br-Mmc) were dissolved in ethyl acetate in a molar ration

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of 1:3. The final volume was 25 ml (ca, 1 mg Ado-Cys). After addition of 200 mg of finely
powdered K₂CO₃, the solution was refluxed for 1 hour using a ml-refluxer at 80°C. After
cooling, the reaction solution was injected into a C18 column (Econosphere, C18, 7x300 mm,
Alltech, Dearfield, IL) for separation using HPLC. The fluorescently labeled Ado-Cys was

5   eluted by a linear gradient of methanol; water from 20 to 100% in 30 min. The flow rate was 2
ml/min.

Method 4

Ado-Cys was dissolved in carbonate buffer, pH 9.0 in 1 mM concentration. Fluorescein
isotiocyanate (FITC) (PcPierce, Rockford, IL) was dissolved in DMSO in 5 mM concentration,
10   and diluted to 1 mM with carbonate buffer, pH 9.0. Equal volumes of Ado-Cys and FITC in
carbonate buffer were mixed and incubated in room temperature for 1 hour. The Ado-Cys-
FITC conjugate was then isolated by HPLC using a C18 column (Econsphere, C18, Alltech,
Deerfield, IL). The elution was monitored at UV 260 nm and fluorescence at Ex484 nm and
Em520 nm. The mobile phases were water and methanol in a linear gradient from 0 to 80% of
15   methanol in 35 min.

Coating mutant SAH hydrolase on microtiter well (96 well plate)

Mutant SAH hydrolase (F302S) was coated on flat-bottomed 96 well plate (Dynex
Technologies, Chantilly, Virginia). 200 µl of 20 µg/ml of F302S mutant hydrolase in 50 mM
sodium phosphate buffer, pH 7.6. was added to each well. After incubation at 4°C overnight,
20   the plate was emptied by inversion. After blocking with 0.5% BSA, the plate was then washed
three times with 10 mM PBS containing 0.1 NaCl and 0.05% of Tween 20. After inversion and
tapping, the plate was stored at 4°C before use.

Preparation of standard samples and chemical reagents

1.   Construction of a standard Hcy curve

25   Human albumin (Fraction V powder, Sigma) was dissolved in PBS in a protein
concentration equal to that of human plasma. To 10 ml of the albumin was added 4 ml of 1%
tri-n-butylphosphine (TBP). The mixture was incubated at room temperature for 15 min.
followed by gel filtration through a size exclusion column (Sephacryl-S100, 2 x 90 cm). The
albumin protein concentration was normalized to human plasma concentration using protein
30   concentrator (Bio-Rad). The protein concentration was determined by Bradford reagent (Bio-

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Rad). A series of known concentration of L-homocysteine and L-homocystine were spiked into the TBP-treated human albumin in a final concentrations ranging from 0 to 50 μM. After incubation at 37°C for 1 hour, the L-homocysteine spiked albumin and L-homocystine albumin were aliquoted in 70 μl/tube as standard samples, and stored at -20°C before use.

5   **2.   Wild type SAH hydrolase solution**

The wild type SAH hydrolase (20 mU/50 μl) was dissolved in 50 mM phosphate buffer, Ph 7.2, containing 1 mM EDTA, 0.25 mM Ado and 1 mg/ml of BSA.

        3.   Tri-n-butylphosphine (TBP) solution

Tri-n-butylphoshine (Sigma) was dissolved in dimethylformamide (DMF) to 1%

10   concentration.

        4.   Fluorophore-labeled Ado-Cys solution

Br-Mmc-labeled Ado-Cys or FITC-labeled Ado-Cys was dissolved in 50 mM phosphate buffer, pH 7.2, in a concentration of 0.5 mM.

        5.   SAH hydrolase inhibitor solution

15   Neplanocin A (natural product), an inhibitor of SAH hydrolase, and a substrate of adenosine deaminase, was dissolved in 50 mM phosphate buffer, pH 7.2. The inhibitor solution (50 μM) was used in an enzyme to inhibitor ratio of 1:1.5.

        6.   Multi-enzyme solution

Adenosine (0.2 U/μl), nucleoside phosphorylase (0.2 U/l) and xanthine oxidase (0.2

20   U/μl) were dissolved in 50 mM potassium phosphate buffer, pH 7.2. All the enzymes were from Sigma.

        7.   Washing solution

The plate washing solution contains of 10 mM PBS, pH 7.2, 0.1 M NaCl, and 0.05% Tween 20.

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EXAMPLE 4

Assays of Hcy using the mutant SAH enzyme

Plasma Hcy assay procedure 1

Step 1. Conversion of Hcy to SAH

5   To 50 μl of plasma sample in microcentrifuge tube or in uncoated 96-well plate was

added 20 μl of 1% TBP and 50 μl of the wild type SAH hydrolase solution. After incubation at

25°C for 15 min, 20 μl of the enzyme inhibitor solution was added to the reaction mixture, and

incubated for 10 min. to inactivate SAH hydrolase.

Step 2. Removal of remaining Ado and enzyme inhibitor

10   To the solution in Step 1 was added 30 μl of the multi-enzyme solution, and incubated

for 15 min at room temperature.

Step 3. Trapping the formed SAH onto the mutant SAH hydrolase

150 μl solution in Step 2 was transferred to a microtiter well pre-coated with mutant

SAH hydrolase. After 30 min. incubation at room temperature, the solution was emptied by

15   inversion.

Step 4. Washing

The plate from Step 3 was washed three times with the washing solution followed by

inversion and tapping.

**Step 5. Binding of fluorophore-labeled Ado-Cys to the mutant
         enzyme**

20

100 μl of the fluorophore-labeled Ado-Cys or fluorophore-labeled Ado-5' ester was

added to the microtiter well in Step 4. After 20 min. incubation at room temperature, the plate

was washed three times with the washing solution.

**Step 6. Detection of the mutant SAH hydrolase-bound fluorophore-
         labeled Ado-Cys**

25

To the microtiter well from Step 5, 200 μl of 50 mM phosphate buffer, pH 7.2, was

added, and the plate was read for fluorescence using a plate reader (Molecular Devices, fmax).

The plasma Hcy concentration was calculated from the standard curve constructed under the same conditions.

Alternative Hcy assay

Alternatively, the Hcy assay can also be performed by pre-coating SAH on microtiter
5   well, and using fluorophore-labeled mutant SAH hydrolase for competition binding assay. The details are described as follows:

1.   pre-coating SAH on microtiter well

SAH was conjugated to polylysine by activating the carboxylic group of SAH with PCl_3 at 50°C. The SAH-polylysine conjugate was purified by HPLC, and dissolved in 0.1 M
10   carbonate buffer, pH 9.6. 300 µl of 100 µg/ml SAH-polylysine solution was added to each well, and incubated at 37°C for 6 hours. The plate was then washed three times with washing solution containing 10 mM PBS, 0.1 M NaCl and 0.05% Tween 20. After inversion and tapping, the plate was stored at 4°C before use.

2.   Fluorophore-labeled mutant SAH hydrolase

15   Mutant SAH hydrolase (*e.g.*, F302S) was specifically fluorescence labels on Cys421, an non-essential cysteine residue which is located on the surface of the protein that is not involved in substrate binding and catalysis. Cys421 residue is readily accessible by thiol reactive molecules, and can be modified without effecting the binding affinity of the enzyme. Thiol specific reactive probes such as 7-diethylamino-3(4'-maleimidylphenyl)-4-methylcoumarin
20   (CPM) can specifically label protein thiols. Mutant SAH hydrolase (F302S) (0.5 mg/ml) in 50 mM phosphate buffer, pH 7.2, was incubated with 2 mM of adenine to protect other thiols in the substrate binding site, followed by addition of CPM to final concentration of 50 µM. The reaction mixture was incubated at room temperature for 30 min. followed by gel filtration on a size exclusion column (Sephacryl S-300, 4.5mmx60cm) to remove adenine and excess CPM.
25   The CPM-labeled F302S mutant SAH hydrolase (2 mg/ml) was kept in 50 mM phosphate buffer containing 20% glycerol at -20°C. The comparison of Km (SAH) and Kcat (SAH) for wild type and mutant F302S is shown below in Table 11.

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Table 11.   Comparison of kinetic constants between mutant and wild type SAH
             hydrolases

Enzyme	Km (SAH)	Kcat (SAH)
wild type	7.9 μM	3.8 S^{-1}
F302S	1.0 μM	0.1 S^{-1}

Plasma Hcy assay procedure 2

Step 1. Conversion of Hcy to SAH

To 50 μl of plasma sample in microcentrifuge tube or in uncoated 96-well plate was added 20 μl of 1% TBP and 50 μl of the enzyme inhibitor solution was added to the reaction mixture, and incubated for 10 min. to inactivate SAH hydrolase.

Step 2. Removal of remaining Ado and enzyme inhibitor

To the solution in Step 1 was added 30 μl of the multi-enzyme solution, and incubated for 15 min. at room temperature.

Step 3. Competition binding of SAH to the Mutant SAH hydrolase

One hundred μl of the solution from Step 2 was transferred to a microtiter well pre-coated with polylysine-SAH conjugate to which 150 μl of the fluorophore-labeled mutant SAH hydrolase was added. After incubation at room temperature for 30 min., the plate was inverted and tapped followed by three times of washing with the washing solution.

Step 4. Detection of the fluorophore-labeled mutant SAH hydrolase bound to the microtiter well

To the plate from Step 3 was added 200 μl of 10 nM PBS, and the plate was read by a plate reader (Molecular Devices, fmax) at Ex390 nm and Em460 nm. The plasma concentration of Hcy was calculated from the standard curve constructed under the same conditions with the standard samples.

EXAMPLE 5

Determination of folate contents in serum and erythrocytes

Sample Preparation

Serum folate, which exists primarily as methyltetrahydrofolic acid (Me-THF) is readily determined by a Me-THF-trapping enzyme such as mutant forms of thymidylate synthase,

methionine synthase, dihydrofolate reductase, or folylpolyglutamate synthetase. In contrast, erythrocyte folate exists as polyglutamate derivatives and have to be treated with conjugase to convert folylpolyglutamates to folate before quantitation with mutant folate trapping enzyme. Different forms of folates are converted to one form using folate interconverting enzymes

5   including dihydrofolate reductase, tetrahydrofolate methyltransferase, methylenetetrahydrofolate reductase, thymidylate synthase, methionine synthase. Any one of these enzymes can be chosen for preparation of a folate trapping enzyme using, for example site-directed mutagenesis of nucleic acid that encodes the enzyme.

Preparation of folate trapping enzymes

10   a.   **Mutation of thymidylate synthase**

Glutamine 214 of human thymidylate synthase is highly conserved in all thymidylate synthases and is postulated to interact with nucleotide ligands that bind at the active site. Mutation of Glu214 to serine results in attenuated catalytic activity of the enzyme but retains substrate binding ability. Residue Asn 229 is involved in formation of hydrogen bonds to

15   constrain the orientation of dUMP in binary complexes with dUMP, and in ternary complexes with dUMP and cofactor 5,10-methylenetetrahydrofolate. Mutation of Asn 229 to Ala results in a 2000-fold decrease in the Kcat of the enzyme with a modest increase in Km and Kd. In addition, mutation of His 199 to any other amino acid results reduced catalytic activity of the enzyme. The C-terminal residues of thymidylate synthase are involved in the enzyme catalysis.

20   Mutation of these residues results in attenuated enzyme activity, but retains the substrate or cofactor binding affinity.

b.   Mutation of dihydrofolate reductase

Mutation of Arg 43 to Ala or Trp 21 to His results in a folate-trapping enzyme.

c.   Mutation of folylpolyglutamate synthetase

25   The C-terminal domain (aa's 300-425) of folypolyglutamate synthetase is involved in the folate-binding site of the enzyme. Mutation of Gln421 to Ser leads to an interruption of hydrophobic interactions in the C-terminal domain and results in decreased catalytic activity, but substantially retains substrate-binding ability of the enzyme.

Binding of folate to folate trapping enzyme

Folate in serum is incubated with a folate trapping enzyme, such as Asn 229-thymidylate synthase, which has been precoated on a 96-well plate. After 30 minutes of incubation at room temperature, the plate is washed three times with PBS buffer. Fluorescein-

5   labeled folate is then added to the plate as competitor tracer. The plate is incubated for another 30 min at room temperature.

Detection of bound folate

After being washed for three times with PBS buffer, the plate is read, using an excitation wavelength Ex of 492 nm and an Em at 515 nm with a fluorescence plate reader. The

10   folate content in serum is calculated based on a folate standard curve prepared and tested under the same conditions using known concentrations of folate.

Since modifications will be apparent to those of skill in this art, it is intended that this invention be limited only by the scope of the appended claims.

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CLAIMS

1.   A method for assaying an analyte in a sample, which method comprises:

a)   contacting the sample with a mutant analyte-binding enzyme, wherein the mutant enzyme has binding affinity for the analyte or an immediate analyte enzymatic conversion product but has attenuated catalytic activity; and

b)   detecting binding between the analyte or the immediate analyte enzymatic conversion product and the mutant analyte-binding enzyme, whereby the presence or amount of analyte in the sample is assessed.

2.   The method of claim 1, wherein the analyte is a small molecule.

3.   The method of claim 2, wherein the small molecule is an inorganic molecule.

4.   The method of claim 3, wherein the inorganic molecule is an inorganic ion.

5.   The method of claim 4, wherein the inorganic ion is selected from the group consisting of a sodium, a potassium, a magnesium, a calcium, a chlorine, an iron, a copper, a zinc, a manganese, a cobalt, an iodine, a molybdenum, a vanadium, a nickel, a chromium, a fluorine, a silicon, a tin, a boron and an arsenic ion.

6.   The method of claim 2, wherein the small molecule is an organic molecule.

7.   The method of claim 6, wherein the organic molecule is selected from the group consisting of an amino acid, a peptide, a nucleoside, a nucleotide, an oligonucleotide , a vitamin, a monosaccharide, an oligosaccharide and a lipid.

8.   The method of claim 7, wherein the amino acid is selected from the group consisting of Ala (A), Arg (R), Asn (N), Asp (D), Cys (C), Gln (Q), Glu (E), Gly (G), His (H), Ile (I), Leu (L), Lys (K), Met (M), Phe (F), Pro (P) Ser (S), Thr (T), Trp (W), Tyr (Y) and Val (V).

9.   The method of claim 7, wherein the nucleoside is selected from the group consisting of adenosine, guanosine, cytidine, thymidine, and uridine.

10.   The method of claim 7, wherein the nucleotide is selected from the group consisting of AMP, GMP, CMP, UMP, ADP, GDP, CDP, UDP, ATP, GTP, CTP, UTP, dAMP, dGMP, dCMP, dTMP, dADP, dGDP, dCDP, dTDP, dATP, dGTP, dCTP and dTTP.

11.   The method of claim 7, wherein the vitamin is a water-soluble vitamin.

5   12.   The method of claim 11, wherein the water-soluble vitamin is selected from the group consisting of thiamine, riboflavin, nicotinic acid, pantothenic acid, pyridoxine, biotin, folate, vitamin B_{12} and ascorbic acid.

13.   The method of claim 7, wherein the vitamin is a fat-soluble vitamin.

14.   The method of claim 13, wherein the fat-soluble vitamin is selected from the
10   group consisting of vitamin A, vitamin D, vitamin E, and vitamin K.

15.   The method of claim 7, wherein the monosaccharide is an aldose or a ketose.

16.   The method of claim 7, wherein the monosaccharide is selected from the group consisting of a triose, a tetrose, a pentose, a hexose and a heptose.

17.   The method of claim 16, wherein:
15   the triose is glyceraldehyde; the tetrose is erythrose or threose;
                the pentose is ribose, arabinose, xylose, lyxose or ribulose;
                the hexose is allose, altrose, glucose, mannose, gulose, idose, galactose, talose or fructose; and.
                the heptose is sedoheptulose.

20   18.   The method of claim 7, wherein the lipid is selected from the group consisting of a triacylglycerol, a wax, a phosphoglyceride, a sphingolipid, a sterol and a sterol fatty acid ester.

19.   The method of claim 18, wherein the triacylglycerol is selected from the group consisting of tristearin, tripalmitin and triolein.

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20.   The method of claim 18, wherein the phosphoglyceride is selected from the group consisting of phosphatidylethanolamine, phosphatidylcholine, phosphatidylserine, phosphatidylinositol and cardiolipin.

21.   The method of claim 18, wherein the sphingolipid is selected from the group consisting of sphingomyelin, cerebrosides and gangliosides.

22.   The method of claim 18, wherein the sterol is cholesterol or stigmasterol.

23.   The method of claim 18, wherein the fatty acid is a saturated fatty acid.

24.   The method of claim 23, wherein the saturated fatty acid is selected from the group consisting of lauric acid, myristic acid, palmitic acid, stearic acid, arachidic acid and lignoceric acid.

25.   The method of claim 18, wherein the fatty acid is an unsaturated fatty acid.

26.   The method of claim 25, wherein the unsaturated fatty acid is selected from the group consisting of palmitoleic acid, oleic acid, linoleic acid, linolenic acid and arachidonic acid.

27.   The method of claim 2, wherein the small molecule analyte is homocysteine (Hcy) and the mutant analyte-binding enzyme is a mutant Hcy-binding enzyme, the mutant enzyme substantially retains its binding affinity or has enhanced binding affinity for Hcy or an immediate Hcy enzymatic conversion product but has attenuated catalytic activity.

28.   The method of claim 27, wherein the attenuated catalytic activity is caused by mutation in the mutant enzyme's catalytic site, its binding site for its co-enzyme, co-factor, non-Hcy substrate, or a combination thereof.

29.   The method of claim 27, wherein the mutant enzyme is a mutant cystathionine ß-synthase and the attenuated catalytic activity is caused by mutation in the mutant cystathionine ß-synthase's catalytic site, its binding site for pyridoxal 5'-phosphate or L-serine, or a combination thereof.

30. The method of claim 27, wherein the mutant enzyme is a mutant methionine synthase and the attenuated catalytic activity is caused by mutation in the mutant methionine synthase's catalytic site, its binding site for vitamin B_{12} or 5-methyltetrahydrofolate (5-CH_3-THF), or a combination thereof.

31. The method of claim 30, wherein the mutant methionine synthase is *E.coli.* methionine synthase comprising one or more mutations selected from the group consisting of His759Gly, Asp757Glu, Asp757Asn, and Ser810Ala.

32. The method of claim 27, wherein the mutant enzyme is a mutant SAH hydrolase, the mutant SAH hydrolase substantially retains its binding affinity or has enhanced binding affinity for Hcy or SAH but has attenuated catalytic activity.

33. The method of claim 32, wherein the attenuated catalytic activity is caused by mutation(s) in the mutant SAH hydrolase's binding site for NAD^+ or NADH or mutation(s) in the mutant SAH hydrolase's catalytic site, or a combination thereof.

34. The method of claim 32, wherein the mutant SAH hydrolase has attenuated 5'-hydrolytic activity but substantially retains its 3'-oxidative activity.

35. The method of claim 32, wherein the mutant SAH hydrolase comprises a sequence of amino acids encoded by the sequence of nucleotides set forth in SEQ ID No. 2 but comprises one or more mutations selected from the group consisting of Phe 302 to Ser (F302S), Lys 186 to Ala (K186A), His 301 to Asp (H301D), His 353 to Ser (H353S), Arg 343 to Ala (R343A), Asp 190 to Ala (D190A), Phe 82 to Ala (F82A), Thr 157 to Leu (T157L), Cys 195 to Asp (C195D), Asn 181 to Asp (N181D), and deletion of Tyr 432 (Δ432).

36. The method of claim 32, wherein the mutant SAH hydrolase comprises the sequence of amino acids set forth in SEQ ID No. 2 except that Arg 431 is replaced by Ala (R431A) and Lys 426 is replaced by Arg (K426R).

37. The method of claim 32, wherein prior to the contact between the sample and the mutant SAH hydrolase, oxidized or conjugated Hcy in the sample is converted into reduced Hcy.

38. The method of claim 32, wherein prior to the contact between the sample and the mutant SAH hydrolase, the Hcy in the sample is converted into SAH.

39. The method of claim 38, wherein the Hcy in the sample is converted into SAH by a wild-type SAH hydrolase.

5   40. The method of claim 39, wherein the SAH is contacted with the mutant SAH hydrolase in the presence of a SAH hydrolase catalysis inhibitor.

41. The method of claim 32, wherein the SAH is contacted with the mutant SAH hydrolase in the presence of a labelled SAH or a derivative or an analog thereof, whereby the amount of the labeled SAH bound to the mutant SAH hydrolase inversely relates to amount of

10   the SAH in the sample.

42. The method of claim 41, wherein the labelled SAH derivative or analog is fluorescently labeled.

43. The method of claim 32, wherein the mutant SAH hydrolase is a labelled mutant SAH hydrolase.

15   44. The method of claim 43, wherein the labelled mutant SAH hydrolase is a fluorescence-labelled mutant SAH hydrolase.

45. The method of claim 27, wherein the mutant enzyme is a mutant betaine-homocysteine methyltransferase and the attenuated catalytic activity is caused by mutation in the mutant betaine-homocysteine methyltransferase's binding site for betaine, its catalytic site,

20   or a combination thereof.

46. The method of claim 27, wherein the mutant enzyme is a mutant methioninase and the attenuated catalytic activity is caused by mutation in the mutant methioninase's binding site for R'SH, its catalytic site, or a combination thereof.

47. The method of claim 2, wherein the small molecule analyte is a folate species

25   and the mutant analyte-binding enzyme is a mutant folate-species-binding enzyme, the mutant

enzyme substantially retains its binding affinity or has enhanced binding affinity for the folate species but has attenuated catalytic activity.

48.   The method of claim 47, wherein the folate species is 5,-methyl-tetrahydrofolate, the mutant folate-species-binding enzyme is a mutant methionine synthase, and the attenuated catalytic activity of the mutant methionine synthase is caused by mutation in its catalytic site, its binding site for vitamin B_{12}, Hcy, or a combination thereof.

49.   The method of claim 47, wherein the folate species is tetrahydrofolate, the mutant folate-species-binding enzyme is a mutant tetrahydrofolate methyltransferase, and the attenuated catalytic activity of the mutant tetrahydrofolate methyltransferase is caused by mutation in its catalytic site, its binding site for serine, or a combination thereof.

50.   The method of claim 47, wherein the folate species is 5, 10,-methylene tetrahydrofolate, the mutant folate-species-binding enzyme is a mutant methylenetetrahydrofolate reductase, and the attenuated catalytic activity of the methylenetetrahydrofolate reductase is caused by mutation in its catalytic site.

51.   The method of claim 47, wherein the folate species is 5, 10,-methylene tetrahydrofolate, the mutant folate-species-binding enzyme is a mutant folypolyglutamate synthase, and the attenuated catalytic activity of the folypolyglutamate synthase is caused by mutation in its catalytic site, its binding site for ATP, L-glutamate, Mg^{2+}, or a combination thereof.

52.   The method of claim 47, wherein the folate species is dihydrofolate, the mutant folate-species-binding enzyme is a mutant dihydrofolate reductase, and the attenuated catalytic activity of the mutant dihydrofolate reductase is caused by mutation in its catalytic site, its binding site for NADPH, or a combination thereof.

53.   The method of claim 52, wherein the mutant dihydrofolate reductase is a *Lactobacillus casei* dihydrofolate reductase having an Arg43Ala or Trp21His mutation.

54.   The method of claim 47, wherein the folate species is 5, 10,-methylene tetrahydrofolate, the mutant folate-species-binding enzyme is a mutant thymidylate synthase,

and the attenuated catalytic activity of the mutant thymidylate synthase is caused by mutation in its catalytic site, its binding site for dUMP, or a combination thereof.

55.   The method of claim 54, wherein the mutant thymidylate synthase is a human thymidylate synthase having a mutation selected from the group consisting of Tyr6His,

5   Glu214Ser, Ser216Ala, Ser216Leu, Asn229Ala and His199X, X being any amino acid that is not His.

56.   The method of claim 54, wherein the mutant thymidylate synthase is an *E.coli* thymidylate synthase having an Arg126Glu mutation or a *Lactobacillus casei* thymidylate synthase having a V316Am mutation.

10   57.   The method of claim 2, wherein the small molecule analyte is cholesterol and the mutant analyte-binding enzyme is a mutant cholesterol-binding enzyme, the mutant enzyme substantially retains its binding affinity or has enhanced binding affinity for cholesterol but has attenuated catalytic activity.

58.   The method of claim 57, wherein the mutant cholesterol-binding enzyme is a

15   mutant cholesterol esterase, and the attenuated catalytic activity of the mutant cholesterol esterase is caused by mutation in its catalytic site, its binding site for H_2O or a combination thereof.

59.   The method of claim 58, wherein the cholesterol esterase is a pancreatic cholesterol esterase having a Ser194Thr or Ser194Ala mutation.

20   60.   The method of claim 57, wherein the mutant cholesterol-binding enzyme is a mutant cholesterol oxidase, and the attenuated catalytic activity of the mutant cholesterol oxidase is caused by mutation in its catalytic site, its binding site for O_2 or a combination thereof.

61.   The method of claim 60, wherein the cholesterol oxidase is a *Brevibacterium*

25   *sterolicum* cholesterol oxidase having a His447Asn or His447Gln mutation.

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62.   The method of claim 2, wherein the small molecule analyte is a bile acid or bile salt and the mutant analyte-binding enzyme is a mutant bile-acid-binding or bile-salt-binding enzyme, the mutant enzyme substantially retains its binding affinity or has enhanced binding affinity for the bile acid or bile salt but has attenuated catalytic activity.

5   63.   The method of claim 62, wherein the mutant bile-acid-binding enzyme is a mutant 3-α-hydroxy steroid dehydrogenase, and the attenuated catalytic activity of the mutant 3-α-hydroxy steroid dehydrogenase is caused by mutation in its catalytic site, its binding site for NAD^+ or a combination thereof.

64.   The method of claim 2, wherein the small molecule analyte is glucose and the
10   mutant analyte-binding enzyme is a mutant glucose-binding enzyme, the mutant enzyme substantially retains its binding affinity or has enhanced binding affinity for glucose but has attenuated catalytic activity.

65.   The method of claim 64, wherein the mutant glucose-binding enzyme is a *Clostridium thermosulfurogenes* glucose isomerase having a mutation selected from the group
15   consisting of His101Phe, His101Glu, His101Gln, His101Asp and His101Asn.

66.   The method of claim 64, wherein the mutant glucose-binding enzyme is a mutant hexokinase or glucokinase, and the attenuated catalytic activity of the mutant hexokinase or glucokinase is caused by mutation in its catalytic site, its binding site for ATP or Mg^{2+}, or a combination thereof.

20   67.   The method of claim 64, wherein the mutant glucose-binding enzyme is a mutant glucose oxidase, and the attenuated catalytic activity of the mutant glucose oxidase is caused by mutation in its catalytic site, its binding site for H_2O or O_2, or a combination thereof.

68.   The method of claim 2, wherein the small molecule analyte is ethanol and the mutant analyte-binding enzyme is a mutant ethanol-binding enzyme, the mutant enzyme
25   substantially retains its binding affinity or has enhanced binding affinity for ethanol but has attenuated catalytic activity.

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69.　　The method of claim 68, wherein the mutant ethanol-binding enzyme is a mutant alcohol dehydrogenase, and the attenuated catalytic activity of the mutant alcohol dehydrogenase is caused by mutation in its catalytic site, its binding site for NAD^+ or Zn^{2+}, or a combination thereof.

70.　　The method of claim 69, wherein the mutant alcohol dehydrogenase is a human liver alcohol dehydrogenase having a His51Gln mutation.

71.　　The method of claim 69, wherein the mutant alcohol dehydrogenase is a horse liver alcohol dehydrogenase having a Phe93Trp or Val203Ala mutation.

72.　　The method of claim 2, wherein the small molecule analyte is uric acid and the mutant analyte-binding enzyme is a mutant uric-acid-binding enzyme, the mutant enzyme substantially retains its binding affinity or has enhanced binding affinity for uric acid but has attenuated catalytic activity.

73.　　The method of claim 72, wherein the mutant uric-acid-binding enzyme is a mutant urate oxidase or uricase, and the attenuated catalytic activity of the mutant urate oxidase or uricase is caused by mutation in its catalytic site, its binding site for O_2, H_2O, or copper ion, or a combination thereof.

74.　　The method of claim 73, wherein the mutant urate oxidase is a rat urate oxidase having a mutation selected from the group consisting of H127Y, H129Y and F131S.

75.　　The method of claim 1, wherein the sample is a body fluid or a biological tissue.

76.　　The method of claim 75, wherein the body fluid is selected from the group consisting of urine, blood, plasma, serum, saliva, semen, stool, sputum, cerebral spinal fluid, tears, mucus, amniotic fluid.

77.　　The method of claim 75, wherein the body fluid is blood.

78.　　The method of claim 77, wherein the blood sample is further separated into a plasma or serum fraction.

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79.   The method of claim 75, wherein the biological tissue is selected from the group consisting of connective tissue, epithelium tissue, muscle tissue, nerve tissue, organs, tumors, lymph nodes, arteries and individual cell(s).

80.   The method of claim 41, wherein the labelled SAH, or a derivative or an analog thereof, is immobilized.

81.   The method of claim 1, wherein the mutant enzyme is immobilized.

82.   The method of claim 27, further comprising detecting cholesterol and/or folic acid in the sample.

83.   A combination, comprising:
       a)   a mutant analyte-binding enzyme that retains at least substantially its binding affinity for the analyte or an immediate analyte enzymatic conversion product but has attenuated catalytic activity; and
       b)   reagents for detecting binding between the analyte or the immediate analyte enzymatic conversion product and the mutant analyte-binding enzyme.

84.   The combination of claim 83, wherein the analyte is Hcy.

85.   The combination of claim 84, wherein the reagent for detecting binding between the Hcy or the immediate Hcy enzymatic conversion product and the mutant Hcy-binding enzyme comprises a labelled Hcy, a labelled immediate Hcy enzymatic conversion product, a labelled mutant Hcy-binding enzyme, or a derivative or an analog thereof.

86.   The combination of claim 84, further comprising a reagent for detecting cholesterol and/or folic acid.

87.   A kit, comprising the combination of claim 83.

88.   The kit of claim 87, further comprising instructions for assaying an analyte in a sample.

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89.   An article of manufacture, comprising:

a)   packaging material;

b)   a mutant analyte-binding enzyme that at least substantially retains its binding affinity or has enhanced binding affinity for the analyte or an immediate analyte

5   enzymatic conversion product but has attenuated catalytic activity; and

c)   a label indicating that the mutant analyte-binding enzyme and the means for use in assaying the analyte in a sample.

90.   An isolated nucleic acid fragment, comprising a sequence of nucleotides encoding a mutant S-adenosylhomocysteine (SAH) hydrolase, wherein the mutant SAH

10   hydrolase has at least substantially the same binding affinity (Km) for homocysteine (Hcy) or SAH but has attenuated catalytic activity.

91.   The isolated nucleic acid fragment of claim 90, wherein the attenuated catalytic activity is caused by mutation(s) in the binding site of the mutant SAH for NAD^+ or NADH, or mutation(s) in the mutant SAH hydrolase's catalytic site, or a combination thereof.

15   92.   The isolated nucleic acid fragment of claim 90, wherein the mutant SAH hydrolase has attenuated 5'-hydrolytic activity but substantially retains its 3'-oxidative activity.

93.   The isolated nucleic acid fragment of claim 90, wherein the mutant SAH hydrolase irreversibly binds SAH.

94.   The isolated nucleic acid fragment of claim 90, wherein the mutant SAH

20   hydrolase has a Km for SAH that is about or less than 10.0 μM.

95.   The isolated nucleic acid fragment of claim 90, wherein the mutant SAH hydrolase has one or more insertion, deletion, or point mutation(s).

96.   The isolated nucleic acid fragment of claim 90, wherein the mutant SAH hydrolase comprises amino acids encoded by the sequence of nucleotides set forth in SEQ ID

25   No. 1, except that it comprises one or more mutations selected from the group consisting of mutations that result in a change in Phe 302 to Ser (F302S), Lys 186 to Ala (K186A), His 301 to Asp (H301D), His 353 to Ser (H353S), Arg 343 to Ala (R343A), Asp 190 to Ala (D190A),

Phe 82 to Ala (F82A), Thr 157 to Leu (T157L), Cys 195 to Asp (C195D) and Asn 181 to Asp (N181D), or a deletion of Tyr 432 (Δ432).

97.   The isolated nucleic acid fragment of claim 90, wherein the mutant SAH hydrolase comprises the sequence of amino acids encoded by the sequence of nucleotides set

5   forth in SEQ ID No. 1, except that it comprises Arg 431 to Ala (R431A) and Lys 426 to Arg (K426R) mutations.

98.   The isolated nucleic acid fragment of claim 90, wherein the nucleic acid is DNA.

99.   The isolated nucleic acid fragment of claim 90, wherein the nucleic acid is
10   RNA.

100.   A plasmid, comprising the nucleic acid fragment of claim 90.

101.   A cell, comprising the plasmid of claim 100.

102.   The cell of claim 101 selected from the group consisting of a bacterial cell, a yeast cell, a fungal cell, a plant cell, an insect cell and an animal cell.

15   103.   A method for producing a mutant SAH hydrolase, comprising: growing the cell of claim 101 under conditions whereby the mutant SAH hydrolase is expressed by the cell; and recovering the expressed mutant SAH hydrolase.

104.   A substantially purified mutant SAH hydrolase that at least substantially retains its binding affinity for homocysteine (Hcy) or SAH, but has attenuated catalytic activity.

20   105.   The substantially purified mutant SAH hydrolase of claim 104, wherein the attenuated catalytic activity is caused by mutation(s) in the mutant SAH hydrolase's binding site for NAD$^+$, or mutation(s) in the mutant SAH hydrolase's catalytic site, or a combination thereof.

106.   The substantially purified mutant SAH hydrolase of claim 104, wherein the mutant SAH hydrolase has attenuated 5'-hydrolytic activity but substantially retains its 3'-oxidative activity.

107.   The substantially purified mutant SAH hydrolase of claim 104, wherein the mutant SAH hydrolase irreversibly binds SAH.

108.   The substantially purified mutant SAH hydrolase of claim 104, wherein the mutant SAH hydrolase comprises the sequence of amino acids set forth in SEQ ID No. 1 but has one or more mutations selected from the group consisting of Phe 302 to Ser (F302S), Lys 186 to Ala (K186A), His 301 to Asp (H301D), His 353 to Ser (H353S), Arg 343 to Ala (R343A), Asp 190 to Ala (D190A), Phe 82 to Ala (F82A), Thr 157 to Leu (T157L), Cys 195 to - Asp (C195D), Asn 181 to Asp (N181D), and deletion of Tyr 432 (Δ432).

109.   The substantially purified mutant SAH hydrolase of claim 104, wherein the mutant SAH hydrolase comprises the sequence of amino acids set forth in SEQ ID No. 2 but comprises Arg 431 to Ala (R431A) and Lys 426 to Arg (K426R) mutations.

110.   A kit for assessing levels of homocysteine in a sample, comprising:
           the mutant enzyme of claim 104; and
           a reagent for detecting binding of the mutant enzyme to a substrate.

111.   A conjugate, comprising:
           a)   a mutant analyte-binding enzyme, wherein the mutant enzyme has binding affinity for an analyte or an immediate analyte enzymatic conversion product but has attenuated catalytic activity; and
           b)   a facilitating agent linked to the mutant enzyme directly or via a linker, wherein the agent facilitates:
                    i)   affinity isolation or purification of a fusion protein;
                    ii)   attachment of the fusion protein to a surface; or
                    iii)   detection of the fusion protein.

112.   The conjugte of claim 111, that comprises a plurality of agents linked thereto.

113.   The conjugate of claim 111, that is a chemical conjugate.

114.   The conjugate of claim 111, that is a fusion protein.

115.   The conjugate of claim 111, wherein the facilitating agent is a protein or peptide fragment.

5   116.   The conjugate of claim 115, wherein the protein or peptide fragment comprises a protein binding sequence.

117.   The conjugate of claim 115, wherein the protein or peptide fragment is selected from the group consisting of an epitope tag or an IgG binding protein, a DNA binding protein, an RNA binding protein, a lipid binding protein, a polysaccharide binding protein, a metal 10   binding protein, an enzyme, a transport protein, a nutrient or storage protein, a contractile or motile protein, a structural protein, a defense protein, a regulatory protein, or a fluorescent protein, and specific binding portions thereof.

118.   The conjugate of claim 117, wherein the DNA binding protein binds to single-stranded or double-stranded DNA molecules.

15   119.   The conjugate of claim 117, wherein the DNA binding sequence binds to DNA that is involved in replication, transcription, DNA repair, recombination, transposition or DNA structure maintenance.

120.   The conjugate of claim 117, wherein the RNA binding protein binds to a single-stranded or double-stranded RNA.

20   121.   The conjugate of claim 117, wherein lipid binding protein binds to a lipid selected from the group consisting of a triacylglycerol, a wax, a phosphoglyceride, a sphingolipid, a sterol and a sterol fatty acid ester.

122.   The conjugate of claim 117, wherein the polysaccharide binding protein binds to starch, glycogen, cellulose or hyaluronic acid.

123.   The conjugate of claim 117, wherein the metal binding protein binds to a metal ion selected from the group consisting of a sodium, a potassium, a magnesium, a calcium, a chlorine, an iron, a copper, a zinc, a manganese, a cobalt, an iodine, a molybdenum, a vanadium, a nickel, a chromium, a fluorine, a silicon, a tin, a boron and an arsenic ion.

5   124.   The conjugate of claim 117, wherein the enzyme is selected from the group consisting of a peroxidase, a urease, an alkaline phosphatase, a luciferase and a glutathione S-transferase.

125.   The conjugate of claim, 111, wherein the mutant analyte-binding enzyme is a mutant SAH hydrolase that at least substantially retains its binding affinity for Hcy or SAH, but

10   has attenuated catalytic activity.

126.   An isolated nucleic acid fragment, comprising a sequence of nucleotides encoding a fusion protein of claim 114.

127.   The isolated nucleic acid fragment of claim 126, wherein the nucleic acid is DNA.

15   128.   The isolated nucleic acid fragment of claim 126, wherein the nucleic acid is RNA.

129.   A plasmid, comprising the nucleic acid fragment of claim 126.

130.   A cell, comprising the plasmid of claim 129.

131.   The cell of claim 130, wherein the cell is selected from the group consisting of a

20   bacterial cell, a yeast cell, a fungal cell, a plant cell, an insect cell and an animal cell.

132.   A method for producing a fusion protein, comprising: growing the cell of claim 130 under conditions whereby the fusion protein is expressed by the cell; and recovering the expressed fusion protein.

133. A method for assaying an analyte in a sample, comprising:

a) contacting the sample with a conjugate of claim 111; and

b) detecting binding between the analyte or the immediate analyte enzymatic conversion product and the conjugate, whereby the presence or amount of the analyte in the sample is assessed.

134. The method of claim 134, wherein the conjugate is a fusion protein.

135. The method of claim 134, wherein prior to the contact between the sample and the conjugate, the conjugate is isolated or purified through affinity binding between the facilitating agent of the conjugate and an affinity binding moiety specific therefor.

136. The method of claim 134, wherein prior to the contact between the sample and the conjugate, the conjugate is attached to a surface via the facilitating agent.

137. The method of claim 134, wherein the analyte is Hcy and the mutant analyte-binding enzyme of the fusion protein is a mutant Hcy-binding enzyme, the mutant enzyme substantially retains its binding affinity or has enhanced binding affinity for Hcy or an immediate Hcy enzymatic conversion product but has attenuated catalytic activity.

138. The method of claim 2, wherein the small molecules are markers associated with a disease, disorder, defect, condition or infection.

139. The method of claim 2, wherein the small molecule is a drug and the method assesses therapeutic efficacy.

140. A solid support, comprising a plurality of mutant analyte binding enzymes.

141. The support of claim 140, wherein the plurality are arranged in an array, comprising at least three mutant analyte binding enzymes.

142. The support of claim 140, wherein the mutant analyte binding enzyme comprises a conjugate containing a facilitating agent.
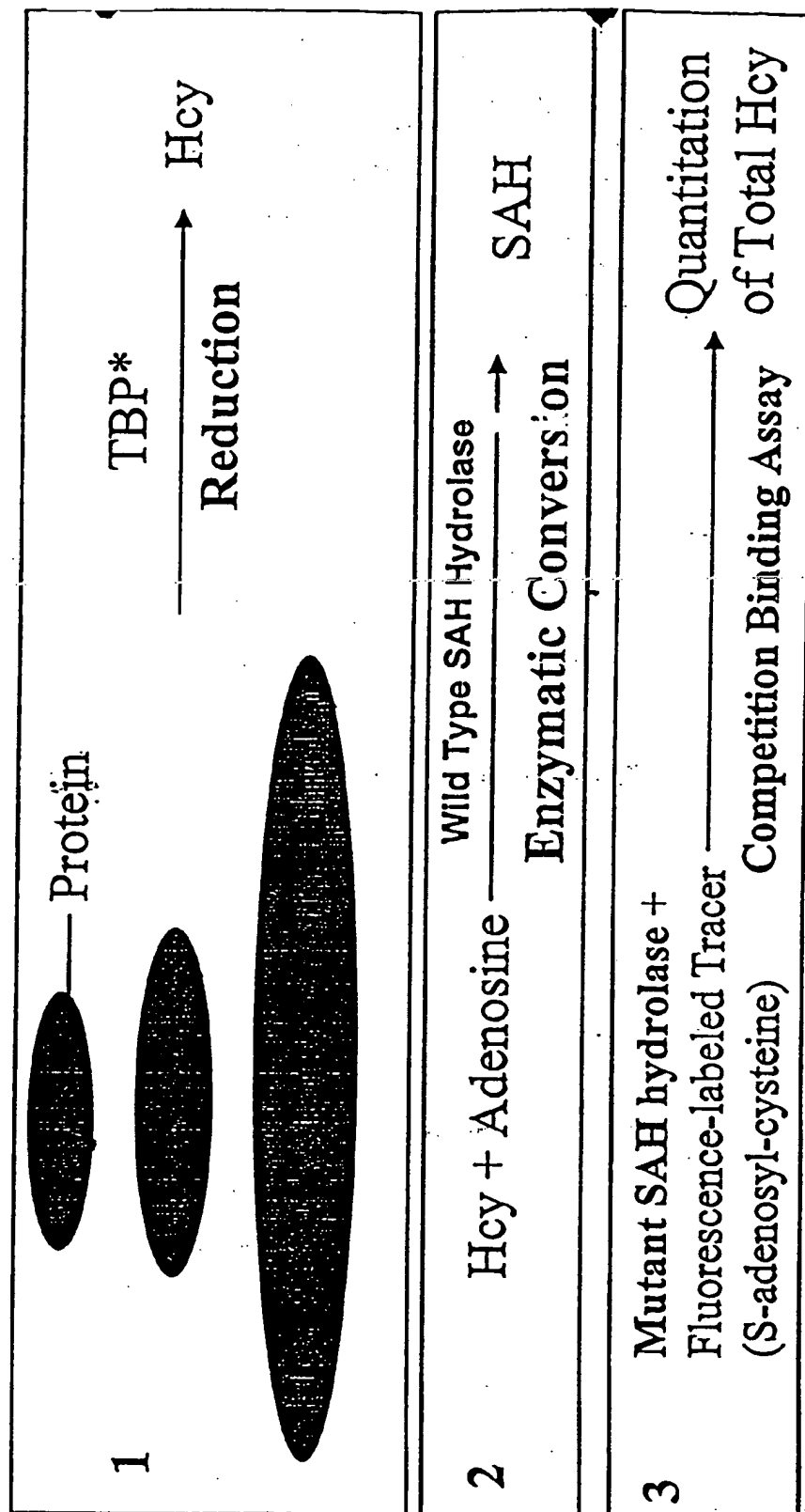
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143.   The support of claim 140 that is a silicon or silicon coated chip.

144.   The support of claim 143, wherein the silicon is derivatized for linking a protein
thereto.

145.   A conjugate, comprising a mutant analyte binding enzyme and a facilitating

5   agent.

FIG. 1

FIG. 2

Design and synthesis of fluorescence-labeled tracer

For Example:

Adenosyl-cysteine

+

4-Bromomethyl-7-methoxycoumarin

refluxing acetone or ethyl acetate

Fluorescence labelled adenosyl-cysteine

Ex=328, Em=359

FIG. 3

FIG. 4